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(54) Title: COMPOSITIONS AND THEIR USES FOR TRANSFER OF DOWN-REGULATORY GENES INTO CELLS ASSOCIATED WITH INFLAMMATORY RESPONSES

(57) Abstract

Genes encoding down-regulatory molecules are transferred into inflammatory cells, including T lymphocytes and dendritic cells. Local inflammatory responses are suppressed by the targeted delivery of such genes. The down-regulatory molecules can be secreted cytokines or cytokine blockers that act extracellularly to suppress inflammation. Alternatively, the down-regulatory molecules act intracellularly to disrupt internal signaling and activation in disease associated lymphocytes. Specificity of targeting is achieved by *ex vivo* gene transfer into disease associated cells, or by the use of retroviral gene transfer vectors having specific targeting envelope proteins. The use of inducible promoters allows local secretion of the cytokines and limits the effect of down-regulatory T cells to sites of antigen recognition.

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COMPOSITIONS AND THEIR USES FOR TRANSFER OF DOWN-REGULATORY GENES INTO CELLS ASSOCIATED WITH INFLAMMATORY RESPONSES

BACKGROUND

The elucidation of the biochemistry of the immune system is one of the great achievements in modern medicine; one that has opened the doors to a much richer understanding of human disease. To a large extent, the story of immunity is the story of lymphocytes. Lymphocytes possess an extremely complex and subtle system for interacting with each other, with antigen-presenting cells, and with foreign antigens and cells. As mobile cells, they utilize numerous receptors and soluble factors.

Modulation of the immune response varies with the specific factors produced, and the receptors present on the responding cell. The pathways for down-regulating responses are as important as those required for activation. T-cell tolerance is one well-known mechanism for preventing an immune response to a particular antigen. Other mechanisms, such as secretion of suppressive cytokines, are also known.

Under some conditions, there can be a failure in down-regulation, resulting in autoimmune disease. Among these diseases are multiple sclerosis and myasthenia gravis, diseases of the joints, such as rheumatoid arthritis, attacks on nucleic acids, as observed with systemic lupus erythematosus and such other diseases as psoriasis, insulin dependent diabetes mellitus (IDDM), Sjogren's disease, and thyroid disease.

The initiating step in autoimmune disease pathology is still mysterious in many cases, particularly in humans

where the diseases are largely sporadic, and symptoms may appear years after the first T cell launches its attack. It has therefore been difficult to design effective therapies that prevent initiation of disease. In many autoimmune diseases the later stages have some common features. Inflammation at the site of the disease is often found, caused by the release of inflammatory cytokines by T cells, and accompanied by the destruction of autologous cells.

Multiple sclerosis is characterized by infiltration of leukocytes into the cerebrospinal fluid, inflammation and demyelination. Rheumatoid arthritis is evidenced in the overt disease by severe inflammation and pain in the affected joints, produced by the malign growth of synovial cells. IDDM leads to destruction of insulin-secreting b cells and overt hyperglycemia. In the NOD mouse, pancreatic inflammatory lesions can be visualized at about four weeks of age. Lymphocytes then begin to invade the islets and destroy b-cells. All of these diseases are marked by inflammatory responses.

Evidence has been presented in the literature for the involvement of different T cell subsets in the development of disease. For example, in EAE, it has been shown that TH-2 type CD4' cells are prevent induction of disease, Weiner *et al.* (1994) Annu. Rev. Immunol. **12**:809-837. It has been shown that TH-1 type CD4+ cells induce severe arthritis in a mouse model, Germann *et al.* (1995) P.N.A.S. **92**:4823-4827. In the NOD mouse, the CD4+ T cell subset appears to contain both pathogenic and protective cell populations, the balance of which changes as diabetes develops. Sempe *et al.* (1994) Diabetologia **37**:337 343 provides evidence of CD4+ regulatory T cells in the NOD male mouse. T cell mediated inhibition of the transfer of autoimmune diabetes in NOD mice is demonstrated in Hutchings and Cooke (1990) J. Autoimmun. **3**:175-185; and

Boitard *et al.* (1989) J. Exp. Med. **169**:1669-1680. Various explanations for this apparent paradox have been put forth, but a definitive answer has not been found. It is possible that pathogenic and protective CD4+ secrete different cytokines. Certain cytokines have been associated with suppression of T cell response. These include IL-10, IL-4 and TGF-b.

The role and identity of antigen presenting cells has been the subject of controversy in the past. Both macrophages and dendritic cells (sometimes referred to as lymphoid interdigitating dendritic cells) are now known to present antigen. Macrophages present to activated T cells and B cells. However, T helper cell priming is dependent on antigen presentation by dendritic cells. It appears that dendritic cells pick up and process antigen in the peripheral blood, then travel into the lymph, and finish maturation in the paracortical T cell zone of lymph nodes. Macrophages and dendritic cells at all levels of maturity can present antigen to preactivated T cells, but only mature dendritic cells are able to prime naive T cells. Dendritic cells are derived from hematopoietic stem cells in the bone marrow. Precursor and immature dendritic cells are found in the blood and lymph. The morphologically distinct, fully mature dendritic cells are found in the spleen and lymph node. These cells have an important role in the induction of inflammatory diseases.

The transcriptional factor NF-kB is active in T cells, and controls the cellular response to certain signaling pathways, along with the expression of proinflammatory cytokines, described in Matsusaka *et al.* (1993) Proc. Natl. Acad. Sci. USA **90**:10193-10197. This activity can be inhibited by a transdominant negative mutant, Logeat *et al.* (1991) EMBO J. **10**(7):1827-32. The inhibitor IkB also inhibits NF-kB, Nolan *et al.* (1991) Cell **64**:961-969. Targeted disruption of NF-kB leads to

multifocal defects in immune responses, Sha *et al.* (1995) Cell **80**:321-330.

An important goal of retroviral gene transfer, and gene therapy in general, has been to deliver genes to specific cell subsets. The use of lymphocytes as cellular vehicles for gene therapy is discussed in Culver *et al.* (1991) Proc. Natl. Acad. Sci. USA **88**:3155-3159. Often, the target cells of interest express cell surface determinants that distinguish them from other cells. In certain cases, ligands have been cloned for these determinants, while in other cases monoclonal antibodies are available. The ability to target retroviruses to specific cellular subsets has been achieved recently. Tissue specific targeting of retroviral vectors through ligand receptor interactions is discussed in Kasahara *et al.* (1994) Science **266**:1373-1375.

Godfrey *et al.* (1994) J. Exp. Med. **180**:757-762 describes the identification of a human OX 40 ligand, a costimulator of CD4+ T cells with homology to tumor necrosis factor. Weinberg *et al.* (1994) J. Immunol. **152**:4712-4721 demonstrates target organ-specific up-regulation of the MRC OX-40 marker, and selective production of lymphokine mRNA by encephalitogenic helper cells isolated from the spinal cord of rats with experimental autoimmune encephalomyelitis.

Many of the clinical symptoms of autoimmune disease could be reduced by preventing inflammation at the site of disease. Immunosuppressive drugs, such as cyclosporin A, have been used in therapy. Their lack of specificity, however, is a severe drawback. Treatment that could specifically inhibit T cell activation at the autoimmune lesion would be of great medical benefit.

SUMMARY OF THE INVENTION

Methods and compositions are provided for the transfer of genes encoding down-regulatory molecules into

cells associated with inflammation. The inflammatory response of T cells associated with disease conditions is suppressed by the targeted delivery of such genes. The suppression of T cell inflammatory responses is useful for treatment of inflammatory conditions The down-regulatory molecules can be secreted cytokines or cytokine blockers that act to extracellularly to suppress inflammation. Alternatively, the down-regulatory molecules act intracellularly to disrupt internal signaling and activation in the disease associated lymphocytes. Down-regulatory molecules may also be cell surface molecules that induce apoptosis in activated, inflammatory T cells.

Specificity of targeting is achieved by *ex vivo* gene transfer into disease associated lymphocytes, or by the use of retroviral gene transfer vectors specific for cells associated with inflammation. The use of inducible promoters allows local secretion of the cytokines and limits the effect of down-regulatory gene expression to sites of antigen recognition.

Brief Description of the Drawings

Figures 1A to 1D are schematics of retroviral genomes. Figure 1A is an expression construct for a down-regulatory gene. Figure 1B further includes a neomycin resistance marker and Internal Ribosome Entry Site (IRES). Figure 1C is a self-inactivating virus. Expression of the down-regulatory gene is controlled by an NFAT/IL-2 minimal promoter. Figure 1D further includes a neomycin resistance marker and Internal Ribosome Entry Site (IRES).

Figure 2 is a schematic of a construct used to generate high titer retrovirus supernatants. The retroviral genome is inserted into an episomal vector having an origin of replication from the Epstein-Barr

virus, Epstein-Barr nuclear antigen (EBNA), and selectable markers.

Figures 3A to 3B are schematics of constructs to be stably incorporated into a packaging cell line. The retroviral envelope protein is shown fused with a ligand for the activated T cell marker OX-40. Figure 3C shows the site for fusion in the DNA sequence of an ecotropic envelope protein. Figure 3D shows the site for fusion in the DNA sequence of a xenotropic envelope protein.

Figure 4A shows a packaged retrovirus particle having a broad specificity envelope protein. Figure 4B shows a retrovirus particle having a mixture of envelope proteins on its surface, which provides for targeting specificity.

Figure 5 is a graph showing the proliferative response of an insulinoma-specific T cell line in response to whole-cell extracts of islets, insulinoma, and pancreatic hormones. T cell hybridomas generated from the cell line were assayed for release of IL-2.

Figure 6 is a bar graph showing cytokine release profiles from CD45RB low $CD4^+$ splenocytes from NOD mice that were diabetic, young non-diabetic, old non-diabetic, or from a control strain.

Figure 7 depicts the incidence of diabetes after transfer of CD45RB low $CD4^+$ splenocytes from diabetic or older non-diabetic NOD mice into NOD-scid recipients.

Figures 8A to 8C are schematics of retroviral vectors encoding dominant negative NF-kB and IkB mutant proteins.

Figure 9 shows a Western blot analysis of expression of mutant IkB-a protein.

Figure 10 illustrates the domains of retroviral envelope protein, and their role in viral infection and specificity.

Figure 11 is a graph showing protection by cells transfected with an IL-4 DRV.

ABBREVIATIONS

Down-regulatory vector (DRV). Down-regulatory sequence (DRS). Self-inactivating down regulatory vector (SIN-DRV). Dominant negative signaling (DNS). Long terminal repeat (LTR). Chimeric retargeting envelope (CREnv). Envelope protein (env). Nuclear factor of activated T cells (NF-AT). Moloney murine leukemia virus (MMLV). Internal ribosome entry site (IRES). Dendritic cell (DC). Insulin dependent diabetes mellitus (IDDM). Rheumatoid arthritis (RA). Reactive arthritis (ReA). Multiple sclerosis (MS). American type culture collection (ATCC). Interleukin (IL). Tumor growth factor (TGF). Tumor necrosis factor (TNF). Antibody (Ab).

DATABASE REFERENCES FOR NUCLEOTIDE AND AMINO ACID SEQUENCES

The complete mRNA sequence of the human OX-40 ligand has the Genbank accession number X79929.

The complete DNA sequence of the human IL-10 gene has the Genbank accession number X78437. The amino acid sequence has Swissprot accession number P22301. The amino acid sequence of murine IL-10 has Swissprot accession number P18893. The complete mRNA sequence of murine IL-10 has the Genbank accession number M37897. The complete DNA sequence of the human IL-4 gene has the Genbank accession number M23442. The complete mRNA sequence has the Genbank accession number M13982. The amino acid sequence has the Swissprot accession number P05112. The amino acid sequence of murine IL-4 has the Swissprot accession number P07750.

The amino acid sequence of human TGF b1 has the Swissprot accession number P01137. The amino acid sequence of human TGF b2 has the Swissprot accession number P08112.

The amino acid sequence of human TGF b3 has the Swissprot accession number P10600.

The complete cDNA sequence of human CTLA-4 has the Genbank accession number L15006. The region of amino acids 1-37 is the leader peptide; 38-161 is the extracellular V-like domain; 162-187 is the transmembrane domain; and 188-223 is the cytoplasmic domain. Variants of the nucleotide sequence have been reported, including a G to A transition at position 49, a C to T transition at position 272, and an A to G transition at position 439. The complete DNA sequence of mouse CTLA-4 has the EMBL accession number X05719 (Brunet *et al.* (1987) Nature **328**:267-270). The region of amino acids 1-35 is the leader peptide.

The complete DNA sequence of human B7-1 (CD80) has the Genbank accession number X60958; the accession number for the mouse sequence is X60958; the accession number for the rat sequence is U05593. The complete cDNA sequence of human B7-2 (CD86) has the Genbank accession number L25259; the accession number for the mouse sequence is L25606.

The genes encoding CD28 have been extensively characterized. The chicken mRNA sequence has the Genbank accession number X67915. The rat mRNA sequence has the Genbank accession number X55288. The human mRNA sequence has the Genbank accession number J02988. The mouse mRNA sequence has the Genbank accession number M34536.

The complete mRNA sequence of the human fas ligand has the Genbank accession number U24231.

Detailed Description of the Invention

The pro-inflammatory response of activated T cells in a mammalian host is decreased following administration of retrovirus vectors comprising T cell down-regulatory genes. The retrovirus infects inflammation associated cells, specifically T cells and dendritic cells. On infection and integration, the down-regulatory DNA

sequences encoded by the retrovirus are expressed. The encoded proteins act to decrease local inflammatory responses, such as those associated with autoimmune disease, graft rejection, *etc.* The retrovirus can be administered to a patient as packaged virus particles, or in the provirus form, *i.e.* integrated DNA in T cells or dendritic cells. Specificity is provided by engineering of the viral envelope protein, and by the use of regulated promoters. The down-regulatory molecules are secreted cytokines or cytokine blockers that act to locally suppress inflammation, intracellular proteins that disrupt internal signaling and activation in disease associated lymphocytes, or cell surface molecules that induce apoptosis of activated T cells.

The subject methods provide a means for therapeutic treatment and scientific investigation of conditions associated with inflammation. The onset of disease is delayed or prevented, and disease progression may be reversed after initial clinical symptoms have appeared. The down-regulatory vectors (DRV) find use in the treatment of autoimmune diseases characterized by the involvement of pro-inflammatory T cells. Other conditions that may be treated include inflammation of the central nervous system caused by bacterial and viral infection, including inflammatory response to vaccination, local inflammation in response to trauma, graft rejection, and other conditions associated with pathogenic T cell activity. Animal models, particularly small mammals, *e.g.* murine, lagomorpha, *etc.* are of interest for experimental investigations. The DRV find use *in vitro* for drug screening assays and the like.

A common feature in a number of diseases and inflammatory conditions is the involvement of pro-inflammatory $CD4^+$ T cells. These T cells are responsible for the release of inflammatory, Th-1 type cytokines at the site of autoimmune tissue destruction.

Cytokines characterized as Th-1 type include interleukin 2 (IL-2), g-interferon, TNFa, IL-12, *etc.* Such pro-inflammatory cytokines act to stimulate the immune response, in many cases resulting in the destruction of autologous tissue. The subject therapy reduces the inflammatory activity of such T cells by decreasing the local concentration of active pro-inflammatory cytokines, through introduction of down-regulatory genes in a retroviral expression vector.

Nucleic Acid Sequences in Down-Regulatory Vectors

The subject retrovirus constructs provide for expression of down-regulatory genes in infected cells. An advantage of retroviruses is that their integration into the host genome allows for their stable transmission through cell division. This ensures that in cell types which undergo multiple independent maturation steps, such as hematopoietic cells, the retrovirus construct will remain resident and continue to be expressed.

Figure 1A illustrates the basic form of a down regulatory-vector (DRV). Inserted into the vector is a down-regulatory sequence (DRS). The DRS encodes a protein that suppresses proinflammatory responses by activated T cells. The DRS may encode a soluble cytokine or cytokine blocker, an intracellular dominant negative signaling molecule or cell surface molecules that induce apoptosis of activated T cells. Cytokines and cytokine blockers are secreted, soluble molecules that are able to affect the activity of nearby cells. Dominant negative signaling molecules of interest are localized inside the cell, and therefore act only on an infected cell. Apoptosis is induced by the intercellular interaction between activated T cells, which constitutively express the fas gene (CD95), and the fas ligand (CD95L). Generally, the DNS vectors will only be used to infect T cells, while vectors encoding

cytokines, cytokine blockers and fas ligand may be used to infect either T cells or dendritic cells. These differences are considerations that will be considered below in the specific construction of the DRV. The length of the inserted DNA is constrained by packaging requirements to be not more than about 7000 nucleotides, therefore it is desirable that the packaged DRS be a cDNA, *e.g.* lacking introns. However, plasmids and other DNA vectors that are used to generate the retroviral RNA genome may contain longer sequences that are subsequently spliced into suitably sized RNA.

Down-regulatory cytokines of interest include interleukin 10 (IL-10); interleukin 4 (IL-4); TGF-b; and the like. The DRS may comprise all or a portion of the coding region of the mature cytokine, and will include the native or an exogenous signal sequence to provide for secretion of the cytokine. Where a portion of the coding sequence is used, that portion will be sufficient to provide substantially all of the normal activity of the cytokine, usually at least about 50% of the normal activity on a molar ratio. Various measures of cytokine activity are known. Specific examples include the measurement of proliferation of the cell line TF-1 in response to IL-4 (Kitamura *et al.* (1989) J. Cell. Physiol. **140**:323); proliferation of the mast cell line MC/9 in response to IL-10 (Thompson-Snipes (1991) J. Exp. Med. **173**:507); and inhibition of IL-4 dependent ^{3}H-thymidine uptake in HT-2 cells by TGF-b1 (Tsang *et al.* (1990) Lymphokine Res. **9**:607). Purified cytokines for use as a control in activity assays are commercially available (see, for example R & D Systems).

The subject cytokines may be modified in a variety of ways. Sequence analogs may be prepared by oligopeptide synthesis using a stepwise substitution of the amino acids at each position with alanine or valine, particularly

alanine. Generally the total number of amino acids substituted will not exceed 3, ranging from 1 to 3, usually 1 to 2. Methods of producing "scanning" mutations are known in the art, and have been successfully used with a number of different peptides. Examples of protocols for scanning mutations may be found in Gustin, *et al.* (1993) Biotechniques **14**:22; Barany (1985) Gene **37**:111-23; Colicelli, *et al.* (1985) Mol Gen Genet **199**:537-9 and Prentki, *et al.* (1984) Gene **29**:303-13.

The cytokines may be produced as a fusion protein, where another peptide sequence provides for modulation of stability *in vivo*, localization to the site of disease, and other functional changes. Various peptides may be used, such as the immunoglobulin constant region, *e.g.* IgG Fc, portions of homing receptors, ligands to cell surface receptors, and the like.

The species of origin for the cytokine may be the same or different as the host to be treated. The origin of the cytokine will be selected to minimize the antigenicity of the protein, and maximize the activity. Generally the species will be the same, *e.g.* human genes into humans, mouse into mouse, *etc.* More specifically, murine IL-10 and IL-4 are not active on human cells, and so will not be used to treat human patients, while TGF-b is highly conserved and cross-reactive between species. The coding region sequences for these proteins may be accessed as previously cited.

Cytokine blockers are specific binding proteins, generally secreted soluble proteins, that substantially neutralize the activity of pro-inflammatory cytokines, *e.g.* interferon g, TNFa, IL-12, *etc.* Usually at least about 50% of the inflammatory cytokine activity will be neutralized in a stoichiometric mixture with the blocking protein, more usually at least about 75%, preferably at least about 90%. Binding proteins of interest include antibodies; soluble T

cell receptors; binding peptides generated from combinatorial libraries; soluble forms of the specific cytokine receptors, *e.g.* human IFNg receptor (Swissprot P15260), mouse IFNg receptor (Swissprot P15261), human TNF type I receptor (Swissprot P19438), human TNF type II receptor (GenBank M32315), *etc.* Recombinant DNA methods or peptide synthesis may be used to produce chimeric, truncated, or single chain analogs of the binding protein, where chimeric proteins may provide mixture(s) or fragment(s) thereof, or a mixture of an antibody and other specific binding members.

Cytokine blocking proteins of interest include high affinity antibodies, *i.e.* having a binding affinity of at least about 10^{-6}, usually at least about 10^{-7}. The generation of monoclonal antibodies is well known in the art. In addition, high affinity monoclonal antibodies specific for proinflammatory cytokines of interest have been previously described (Williams *et al.* (1992) P.N.A.S. **89**:9784-9788). Of particular interest is the expression of such an antibody as a single chain, instead of the normal multimeric structure. Single chain antibodies are described in Jost *et al.* (1994) J.B.C. **269**:26267-73, and others. DNA sequences encoding the variable region of the heavy chain and the variable region of the light chain are ligated to a spacer encoding at least about 4 small neutral amino acids, such as glycine and serine. The protein encoded by this fusion allows assembly of a functional variable region that retains the specificity and affinity of the original antibody. A signal sequence from the native protein, or an exogenous source will be included in an expression construct for the single chain antibody.

An apoptosis inducing receptor in activated T cells is the fas ligand. The gene is introduced into activated T cells at the site of inflammation; into inflammation associated cells, e.g. dendritic cells; or into double

negative T cells. Where the therapeutic T cells expressing fas ligand also express the fas receptor, the cells may commit suicide before they are able to act on other cells. In such cases the cells are transduced with an apoptosis protective gene, such as the BCL-2 gene.

Dominant negative signaling (DNS) molecules of interest are cytoplasmic proteins that block intracellular signaling leading to pro-inflammatory cytokine expression. DNS of interest include dominant negative mutations of proteins active in the NF-kB signaling cascade. A molecule of particular interest is NF-kB p50, where the DNA binding region has been deleted or replaced with a non-binding sequence. The DNA binding region includes residues 33 through 54 of the mature protein amino acid sequence. The mutated molecule retains the dimerization motif, and therefore its ability to heterodimerize with other NF-kB rel monomers. NF-kB activity is inhibited by at least about 50% in a stoichiometric mixture with the DNS molecule, more usually at least about 75%, preferably at least about 90%.

Another DNS of particular interest is IkB. IkB is a specific regulator of NF-kB p50:p65 heterodimers. It is associated with NF-kB prior to activation, and must be degraded for signaling to proceed. Mutations in at least one of the regions necessary for its degradation create a dominant negative molecule. The regions required for degradation are phosphoserine residues at positions 32 and 36, and the 39 carboxy terminal amino acids. Substitution or deletion of any of these regions, *e.g.* deletion of the 39 amino acid region, mutation or deletion of residue 32, mutation or deletion of residue 36, *etc.*, prevents degradation. NF-kB activity is inhibited by at least about 50% in a stoichiometric mixture with the DNS molecule, more usually at least about 75%, preferably at least about 90%.

The down regulatory-vector (DRV) includes retroviral sequences that are required for packaging, integration and expression of the inserted down-regulatory genes. The DRV genome is single stranded RNA, while the integrated DRV provirus is double stranded DNA. The DRV is "defective" in its inability to encode viral proteins required for productive infection. Replication of the DRV requires growth in a packaging cell line that provides the *gag*, *pol*, and *env* proteins necessary for completion of the infectious cycle.

The DRV contains a y sequence, which permits packaging of the retroviral genome. The y sequence is the region of the retroviral genome downstream from the 5' LTR, extending into the *gag* coding region (Danos *et al.* (1988) PNAS **85**:6460-6464). It has been found that there is no sharp delineation of functional sequences within this region, but that including more of the native sequence will provide for better packaging efficiency. It is preferred that the gag and y sequences be derived from the same retrovirus species, *e.g.* MMLV, ALV, *etc.*, and that the y be positioned immediately downstream of the 5' LTR.

The sequences at the 5' and 3' termini of the DRV are long terminal repeats (LTR). A number of LTR sequences are known in the art and may be used. These include the MMLV-LTR; HIV-LTR; AKR-LTR; FIV-LTR; ALV-LTR; *etc.* Specific sequences may be accessed through public databases. Various modifications of the native LTR sequences are also known. In general, the R and U5 regions are essential for the promoter activity, while some small deletions or substitutions may be made in U3 region and still retain activity. The packaged retroviral RNA genome is known to exclude the 5'U3 sequence, and the 3'U5 sequence, thereby extending from R to R (see Figures 1A to 1D).

Generally the DRV will have a functional 5' LTR. The 5' LTR acts as a strong promoter, driving transcription of the down-regulatory gene after integration into a target cell genome. For some uses, however, it is desirable to have a regulatable promoter driving expression of the DRS. Where such a promoter is included, the promoter function of the LTR will be inactivated. This is accomplished by a deletion of the U3 region in the 3'LTR, including the enhancer repeats and promoter, that is sufficient to inactivate the promoter function. After integration into a target cell genome, there is a rearrangement of the 5' and 3' LTR, resulting in a transcriptionally defective provirus, termed a "self-inactivating vector" (DRV-SIN). A DRV-SIN has an exogenous promoter in addition to the LTR, generally an inducible promoter, which drives transcription of the DRS. Figures 1C and 1D illustrate a SIN-DRV having an inducible promoter.

Suitable inducible promoters are characterized by the transcriptional activation of a cell specific transcriptional response element in activated T cells. Use of an inducible promoter allows the DRV to be targeted to activated T cells with or without the use of engineered envelope proteins. Regulation of transcriptional activation is the result of interaction between transcriptional activators bound to *cis*-regulatory elements, factors bound to basal transcriptional elements and the activity of transcriptional mediators, or coactivators. The absence or presence of any of these factors may affect the level of transcription. Additionally, factors may be present in an inactive form, where the factors are activated through chemical modification, particularly as the result of a cellular signaling mechanism. In some cases, signaling molecules are able to act directly to activate transcription. Any of

these mechanisms may operate to limit the types of cells in which the promoter is active.

It will be understood by one of skill in the art that very low basal levels of transcription may be present in non-targeted cell types. By transcriptional activation, it is intended that transcription will be increased above basal levels in the target activated T cell by at least about 100 fold, more usually by at least about 1000 fold. An inducible promoter of interest is the NF-ATc (nuclear factor of activated T cells) minimal promoter, having three tandem NF-ATc sites upstream of the interleukin 2 minimal promoter (Fiering *et al.* (1990) Genes and Dev. **4**:1823-1834). Other promoters of interest include the IL-2R, IFNg and IkB promoters. In the absence of T cell induction, *i.e.* binding of the antigen receptor and co-stimulatory molecules, the promoter is substantially inactive, but transcription is rapidly induced upon binding of the T cell antigen receptor.

The DRV will normally include a marker that allows for selection of cells into which the DNA has been integrated, as against cells which have not integrated the DRV. Various markers are known in the art, particularly antibiotic resistance markers, such as resistance to G418 (neomycin), hygromycin, puromycin, and the like. Less conveniently, negative selection may be used, where the marker is the *HSV-tk* gene, which will make the cells sensitive to agents such as acyclovir and gancyclovir. Reporter genes, *e.g.* green fluorescent protein, Lac Z, b-gal, or other surface markers not normally expressed on the cell, may be used as markers to track expression in the DRV.

Inclusion of an Internal Ribosome Entry Site (IRES), as illustrated in Figure 1B and 1D, allows the marker or reporter gene to be coordinately expressed with the down-regulatory gene. Where normally a single protein will be

translated from an mRNA, the addition of an IRES sequence allows additional translational starts. An example of a retroviral construct containing IRES sequences is described in Zitvogel *et al.* (1994) Hum. Gene Ther. 5:1493-1506.

One may insert the DRV sequence into an appropriate episomal vector, *e.g.* plasmid, EBV episome, BAC, YAC, etc., and manipulate the vector by restriction, insertion of the desired gene with appropriate transcriptional and translational initiation and termination regions, and then introduce the plasmid into an appropriate packaging host. The vector may be further modified to include functional entities that find use in the preparation of the construct, amplification, transformation of the host cell, *etc.* At each of the manipulations, one may grow the plasmid in an appropriate host, analyze the construct to ensure that the desired construct has been obtained, and then subject the construct to further manipulation. When completed, the plasmid or excised virus may then be introduced into the packaging host for packaging and isolation of virus particles for use in the genetic modification. Specific episomal constructs used for high titer retrovirus production are discussed in detail below.

Production of Down-Regulatory Vectors

Recombinant helper-free retrovirus production depends on a cell line that produces *cis* necessary viral proteins. This ensures that the virus has the capacity to infect only one target cell and is then incapable of producing or transmitting virus to other cell types. These viral proteins include those encoded by the *gag*, *pol* and *env* genes, where each gene may produce polyproteins that are further processed after translation. A number of suitable packaging cell lines are known in the art, see for example, Mulligan (1993) Science **260**:926-932; Pear *et al.* (1993) P.N.A.S. **90**:8392-8396 and Mann *et al.* (1983) Cell

33:153-159. Packaging cells are transfected with the retroviral DNA of interest by any suitable method, *e.g.* electroporation, $CaPO_4$ precipitation, *etc.* The packaged virus is then collected from the supernatant of the packaging cells.

The host cell specificity of the retrovirus is determined by the envelope protein, *env* (p120). The envelope protein is provided by the packaging cell line, and is not encoded in the DRV itself. Envelope proteins are of at least three types, ecotropic, amphotropic and xenotropic. Retroviruses packaged with ecotropic envelope protein, *e.g.* MMLV, are capable of infecting most murine and rat cell types. Ecotropic packaging cell lines include BOSC23 (Pear *et al.*, *supra.*) Retroviruses bearing amphotropic envelope protein, *e.g.* 4070A (Danos *et al*, *supra.*), are capable of infecting most mammalian cell types, including human, dog and mouse. Amphotropic packaging cell lines include PA12 (Miller *et al.* (1985) Mol. Cell. Biol. 5:431-437); PA317 (Miller *et al.* (1986) Mol. Cell. Biol. 6:2895-2902) GRIP (Danos *et al.* (1988) PNAS 85:6460-6464); and yNXa (described herein). Retroviruses packaged with xenotropic envelope protein, *e.g.* AKR *env*, are capable of infecting most mammalian cell types, except murine cells.

Preferred packaging cell lines are derivatives of human 293 cells (ATCC CRL 1573), which rapidly take up and express high levels of transiently introduced DNA. Examples are the BOSC23 and yNXa packaging lines. Vectors have been previously described that allow rapid establishment of stable retroviral producer DNA as EBV-based episomes within such 293 producer systems (Pear *et al.*, *supra.*)

Figure 2 illustrates a typical episomal vector. The vector will generally include a selectable marker active in the producer cell, a selectable marker active in bacterial

cells, and origins of replication for both types of cells. The DRV is inserted into the vector at a suitable site. Transcription initiated from the retroviral LTR produces the retrovirus genome, which is then packaged by the producer cell.

The episomal production technique rapidly produces very large volumes of high titer retrovirus in the cell supernatant, usually of at least about 10^6 per ml, preferably of at least about 10^7 per ml. For direct injection of virus particles, it is desirable to have virus titers of at least about 10^8 per ml. Useful methods for the concentration of virus include calcium mediated precipitation (Morling and Russell (1995) Gene Therapy 2:504-508); dialysis; affinity chromatography; or chromatography on a nickel column.

Targeting Vectors

Of particular interest for delivering retrovirus *in vivo* is the use of a producer cell line that packages the DRV with a targeting envelope protein, where the envelope is engineered to infect a specific cell type, as described in Kasahara *et al.* (1994) Science 266:1373-1375. For the subject vectors, targeting to activated T cells or to dendritic antigen presenting cells is of interest. Dendritic cells (DC) are hematopoietic cells characterized as expressing class I and class II MHC proteins, CD45, CD33 and, for the most part, CD4. The cells lack expression of most lymphoid and monocytic specific cell markers, *e.g.* CD3, CD11b, CD14, CD16 and CD19. The mature subset of dendritic cells found in blood are characterized by expression of CD11c, high levels of CD33, CD45RO, and the co-stimulatory molecules CD80 and CD86. They are able to present antigen so as to stimulate naive or preactivated T cells. Precursor dendritic cells are CD11c negative, express low levels of CD33, are CD45RA positive, have

little antigen presenting activity, and will differentiate into the mature cells *in vitro* and *in vivo*. The term dendritic cells (DC) shall be intended to mean both the mature and immature cells, unless specifically stated otherwise. Activated T cells, as defined herein, are T cells that have been stimulated through the antigen receptor and one or more co-stimulatory receptors. Conventional assays for T cell activation include release of IL-2 and proliferation.

Targeting is accomplished by insertion of protein domains containing addressing information for cell type specificity, *e.g.* a ligand for a receptor expressed by activated T cells or DC; a counter-receptor for addressins, selectins *etc.*, co-stimulatory receptors such as CTLA-4 and CD28; into the envelope protein, to create a chimeric retargeting envelope (CREnv). The leader region and N terminal residues of ecotropic and xenotropic envelope protein are shown in Figure 3. The fusion envelope protein presents a functional targeting domain, but lacks the capacity to complete the viral infection process. Targeted virus is therefore packaged with a mixture of modified and unmodified envelope proteins, as shown in Figures 4B and 10.

The CREnv is expressed in a producer cell line, such as those previously described. A vector providing for expression of the CREnv is introduced into the producer cell line by any suitable method. The expression vector may be any DNA or RNA element that is stably or transiently maintained in the cell line, *e.g.* retrovirus, EBV episome, SV-40 episome, integrated YAC, and the like. Conveniently, the CREnv is introduced in a retroviral construct, where the LTR acts as a promoter. The modified producer line expresses the normal proteins required for retroviral packaging, in addition to the CREnv.

The specific mixture of envelope proteins used for packaging will depend on the species of the intended target cell. To infect human or other non-murine cells, both the CREnv and envelope will be ecotropic, thereby only infecting human cells that bind to the targeting domain. To infect murine cells, both the CREnv and the envelope will be xenotropic, thereby only permitting infection of murine cells that bind to the targeting domain. Generally, at least about 10% of the total envelope protein present on the virus will be the wild-type.

For targeting to activated T cells, CREnv targeted to the OX-40 receptor are of particular interest. OX-40 is expressed on activated CD4+ T cells, but not on resting T cells or other lymphoid or nonlymphoid tissues. By incorporating a binding domain for the OX-40 receptor in the coat protein of the retrovirus, genes are delivered specifically to activated $CD4^+$ T cells. OX-40 binding domains include the OX-40 ligand, antibodies that specifically bind OX-40 receptor. The heavy and light chain variable regions of antibodies can be expressed as a single linear polypeptide, as described above.

Antibodies that specifically recognize OX-40 receptor may be generated by immunization of suitable animals, followed by immortalization and selection of monoclonal antibody producing hybridomas, as known in the art. Alternatively, the previously described monoclonal antibody L106 (Godfey *et al.*, in Activation Antigens AA10.1, pp. 1157-1160) may be used. The DNA sequences encoding the variable portion of the heavy chain and the light chain are isolated by conventional methods, for example through the use of primers directed to conserved sequences in conjunction with the polymerase chain reaction to amplify the region. Of particular interest is the expression of such an antibody as a single chain, instead of the normal multimeric structure. DNA sequences encoding

the variable region of the heavy chain and the variable region of the light chain are ligated to a spacer encoding at least about 4 amino acids of glycine and/or serine. Appropriate sites for fusion into the envelope proteins are shown in Figures 3A and 3B.

An alternative CREnv for targeting to OX-40 receptor fuses the binding domain of OX 40 ligand to a retroviral envelope gene. The extracellular binding domain of the ligand is contained within a 398 nucleotide region in the human gene (shown in Godfey *et al.* (1994) J.E.M. 180:757-762). The binding domain is contained within a 450 nucleotide region of the mouse gene. The nucleic acid sequences may be accessed as previously cited. The binding region from OX-40 ligand in other species may be determined empirically in binding studies with the receptor, or by analogy to the human and mouse sequences. The nucleotide sequence encoding the binding domain is isolated by conventional technology, for example through the use of restriction endonucleases, PCR amplification, *etc.*, and inserted into the appropriate retroviral envelope protein, as shown in Figures 3A and 3B.

For targeting to dendritic cells, a CREnv of particular interest is specific for CD86. CD86 (B7-2) is expressed at high levels on DC, but is generally absent on non-antigen-presenting cells. By incorporating a binding domain for CD86 in the coat protein of the retrovirus, genes are delivered specifically to DC. CD86 binding domains include its counter-receptors CTLA-4 and CD28, and antibodies that specifically bind CD86. The heavy and light chain variable regions of antibodies can be expressed as a single linear polypeptide, as described above.

A DC CREnv will preferably include the extracellular region of CTLA-4. For human CTLA-4, the region of amino acids 1-37 is the leader peptide; 38-161 is the extracellular V-like domain; 162-187 is the transmembrane

domain; and 188-223 is the cytoplasmic domain. Human CTLA-4 is able to bind both mouse and human CD86. The nucleotide sequence encoding the binding domain is isolated by conventional technology, for example through the use of restriction endonucleases, PCR amplification, *etc.*, and inserted into the appropriate retroviral envelope protein.

Alternatively, a DC CREnv will contain a single chain antibody specific for CD86. Antibodies may be generated by immunization of suitable animals, followed by immortalization and selection of monoclonal antibody producing hybridomas, as known in the art. Alternatively, the previously described monoclonal antibody IT209 (Fagnoni *et al.* (1995) Immunol. 85:467-474) may be used. The construction of the antibody CREnv is performed as described above.

Mature DC are non-dividing cells, and so delivery targeted to CD86 expressing cells *in vivo* will usually require a modification of the DRV. The modified vector is capable of infecting non-dividing cells (DRV-ND). This is accomplished by one of the following methods. The MMLV p15 matrix protein may be altered to substitute the SV-40 nuclear localizing sequence KKKRKV for the wild-type MMLV sequence KKRRWV. Similarly, the nucleocapsid p30 protein may substitute the nuclear localizing sequence for the wild-type MMLV sequence RRRHRE. The two modified proteins may be used separately, or in a combined construct. Other nuclear localizing sequences, such as those from Rel proteins, may also be used. Sequence modifications are accomplished by conventional recombinant DNA methods, for example by PCR-driven primer overlap.

Delivery of Down-Regulatory Vectors as a Provirus

As an alternative to the delivery of retrovirus particles, *i.e.* packaged virus, the DRV is delivered in the form of a provirus integrated into a cell, where the cell

provides for localization to the site of interest, either lesional or intra-lesional. Provirus delivery is suitable for secreted DRS, *e.g.* cytokines and cytokine blockers or for apoptosis inducing genes. Transduction of the delivery cell is performed *in vitro*, generally with isolated cell populations or cell lines, using conventional culture methods. In this way, retrovirus packaged with a broad specificity, *e.g.* amphotropic, envelope may be used.

Delivery cells may be xenogeneic, allogeneic, syngeneic or autologous, preferably autologous, in order to reduce adverse immune responses. Suitable cells are those that localize to the site for treatment after administration to a host animal. Preferred cells are T cells and DC.

Autologous T cells for provirus delivery may be isolated from the site of autoimmune lesions, or surrounding areas, *e.g.* lymph nodes, *etc.* Sites include islets of Langerhans for IDDM, cerebrospinal fluid for MS, synovial fluid for RA, etc. The cells are then selected *in vitro* for T cells reactive with the appropriate autoantigen, *e.g.* myelin basic protein for MS, islet extracts for IDDM, collagen for RA, *etc.* The selection process enhances the specificity of the cells for localization to the site of the autoimmune lesion upon reinfusion. Alternatively, genes encoding selectins, antibodies that specifically bind receptors present in the tissue of interest, and the like may be introduced into the delivery cells to confer specific localization.

Dendritic cells may be isolated from the peripheral blood for use in provirus delivery. Methods of isolating dendritic cells have been described (see, for example Thomas *et al.* (1994) J. Immunol. **153**:4016; Ferbas *et al.* (1994) J. Immunol. **152**:4649; and O'Doherty *et al.* (1994) Immunology **82**:487). The separation may involve density gradient centrifugation, immunoadsorption, *e.g.* beads,

magnetic particles, *etc.*, or flow cytometry. The phenotype of dendritic cells has been described above. DC expressing high levels of CD86 are grown in culture in the presence of GM-CSF. The cells are then infected with retrovirus and reinfused into the patient. If desired, the DC may be pulsed with antigen prior to reintroduction.

As an alternative method of administering apoptosis genes, double negative T cells, *i.e.* T cells that are characterized as $CD3^{lo}$, $CD4^-$, $CD8^-$ are isolated from an autologous or allogeneic donor by flow cytometry, panning, antibody-magnetic bead conjugates, *etc.*, as known in the art. These cells express fas ligand, and are able to induce apoptosis in interactions with activated, pro-inflammatory T cells. The cells may be transfected with an expression vector that encodes a protein domain containing addressing information for cell type specificity, *e.g.* a ligand for a receptor expressed by activated T cells; a counter-receptor for addressins, selectins *etc.* Of particular interest is the cell surface expression of OX-40 ligand, or membrane bound form of an antibody specific for OX-40.

Uses of Down-Regulatory Vectors

Inflammation involves capillary dilation, with accumulation of fluid and migration of phagocytic leukocytes, such as granulocytes and monocytes, to the site of injury or lesion. Inflammation is important in defending a host against a variety of infections, but can also have undesirable consequences in inflammatory disorders. Activated T cells have an important role in inflammation, releasing interferon g and colony stimulating factors that in turn activate phagocytic leukocytes. Antigen presenting cells are involved in the T cell activation process.

Conditions characterized by the undesirable release of pro-inflammatory cytokines include autoimmune diseases, inflammation caused by bacterial and viral infection, including response to vaccination, local inflammation in response to trauma, graft rejection, graft *v.* host disease, and the like. Administration of the subject DRVs decreases the local concentration of active pro-inflammatory cytokines, either by inhibiting the synthesis or release of cytokines by activated T cells, by blocking the activity of existing cytokines, or by eliminating activated T cells through apoptosis. The DRV may be packaged with a retroviral envelope that confers specificity of infection. The DRS may be expressed on a SIN-DRV, where an inducible promoter expressed in activated T cells drives transcription, alone, or in combination with a CREnv for targeting specificity. The DRV may also be introduced in the form of a provirus integrated into a delivery cell.

Autoimmune diseases of interest are associated with T cell mediated tissue destruction. Included are multiple sclerosis (MS), rheumatoid arthritis (RA), reactive arthritis, psoriasis, pemphigus vulgaris, Sjogren's disease, thyroid disease, Hashimoto's thyroiditis, myasthenia gravis, insulin dependent diabetes mellitus (IDDM) as well as many others. Treatment of primates, more particularly humans is of interest, but other mammals may also benefit from treatment, particularly domestic animals such as equine, bovine, ovine, feline, canine, murine, lagomorpha, and the like.

The subject therapy will desirably be administered during the presymptomatic or preclinical stage of the disease, and in some cases during the symptomatic stage of the disease. Early treatment is preferable, in order to prevent the loss of function associated with autoimmune tissue damage. The presymptomatic, or preclinical stage will be defined as that period not later than when there is

T cell involvement at the site of disease, *e.g.* islets of Langerhans, synovial tissue, thyroid gland, *etc.*, but the loss of function is not yet severe enough to produce the clinical symptoms indicative of overt disease. T cell involvement may be evidenced by the presence of elevated numbers of T cells at the site of disease, the presence of T cells specific for autoantigens, the release of perforins and granzymes at the site of disease, response to immunosuppressive therapy, *etc.*

Degenerative joint diseases may be inflammatory, as with seronegative spondylarthropathies, *e.g.* ankylosing spondylitis and reactive arthritis; rheumatoid arthritis; gout; and systemic lupus erythematosus. The degenerative joint diseases have the common feature in that the cartilage of the joint is eroded, eventually exposing the bone surface. Destruction of cartilage begins with the degradation of proteoglycan, mediated by enzymes such as stromelysin and collagenase, resulting in the loss of the ability to resist compressive stress. Alterations in the expression of adhesion molecules, such as CD44 (Swissprot P22511), ICAM-1 (Swissprot P05362), and extracellular matrix protein, such as fibronectin and tenascin, follow. Eventually fibrous collagens are attacked by metalloproteases, and when the collagenous microskeleton is lost, repair by regeneration is impossible. There is significant immunological activity within the synovium during the course of inflammatory arthritis. While treatment during early stages is desirable, the adverse symptoms of the disease may be at least partially alleviated by treatment during later stages. Clinical indices for the severity of arthritis include pain, swelling, fatigue and morning stiffness, and may be quantitatively monitored by Pannus criteria. Disease progression in animal models may be followed by measurement of affected joint inflammation. Therapy for inflammatory

arthritis may combine the subject treatment with conventional NSAID treatment. Generally, the subject treatment will not be combined with such disease modifying drugs as cyclosporin A, methotrexate, and the like.

A quantitative increase in myelin-autoreactive T cells with the capacity to secrete IFN-gamma is associated with the pathogenesis of MS, suggesting that autoimmune inducer/helper T lymphocytes in the peripheral blood of MS patients may initiate and/or regulate the demyelination process in patients with MS. The overt disease is associated with muscle weakness, loss of abdominal reflexes, visual defects and paresthesias. During the presymptomatic period there is infiltration of leukocytes into the cerebrospinal fluid, inflammation and demyelination. Family histories and the presence of the HLA haplotype DRB1*1501, DQA1*0102, DQB1*0602 are indicative of a susceptibility to the disease. Markers that may be monitored for disease progression are the presence of antibodies in the cerebrospinal fluid, "evoked potentials" seen by electroencephalography in the visual cortex and brainstem, and the presence of spinal cord defects by MRI or computerized tomography. Treatment during the early stages of the disease will slow down or arrest the further loss of neural function.

Human IDDM is a cell-mediated autoimmune disorder leading to destruction of insulin-secreting b cells and overt hyperglycemia. T lymphocytes invade the islets of Langerhans, and specifically destroy insulin-producing b-cells. The depletion of b cells results in an inability to regulate levels of glucose in the blood. Overt diabetes occurs when the level of glucose in the blood rises above a specific level, usually about 250 mg/dl.

In humans a long presymptomatic period precedes the onset of diabetes. During this period there is a gradual loss of pancreatic b cell function. The disease progression

may be monitored in individuals diagnosed by family history and genetic analysis as being susceptible. The most important genetic effect is seen with genes of the major histocompatibility locus (*IDDM1*), although other loci, including the insulin gene region (*IDDM2*) also show linkage to the disease (see Davies *et al*, *supra* and Kennedy *et al*. (1995) Nature Genetics 9:293-298). Markers that may be evaluated during the presymptomatic stage are the presence of insulitis in the pancreas, the level and frequency of islet cell antibodies, islet cell surface antibodies, aberrant expression of Class II MHC molecules on pancreatic b cells, glucose concentration in the blood, and the plasma concentration of insulin. An increase in the number of T lymphocytes in the pancreas, islet cell antibodies and blood glucose is indicative of the disease, as is a decrease in insulin concentration. After the onset of overt diabetes, patients with residual b cell function, evidenced by the plasma persistence of insulin C-peptide, may also benefit from administration of the subject polysaccharides in order to prevent further loss of function.

The response of the host immune system to a graft, or of a graft towards the host (GVHD) is reduced by treatment with the subject DRV. Grafts include the transplantation of cells, tissues and organs, such as the transfusion of blood or blood components, the grafting of bone, skin, bone marrow, etc., and the transplantation of tissues of the eye, pancreas, liver, kidney, heart, brain, bowel, lung, etc. Of interest are transplantation of hematopoietic cells, *e.g.* bone marrow, mobilized hematopietic stem cells in peripheral blood, *etc.*, transplantation of kidneys and transplantation of hearts. As used herein, a graft recipient is an individual to whom tissue or cells from another individual (donor), commonly of the same species, has been transferred, particularly

where one or more of the Class I MHC antigens are different in the donor as compared to the recipient. However, in some instances xenogeneic, *e.g.* pig, baboon, *etc.*, tissue, cells or organs will be involved. The graft recipient and donor are generally mammals, preferably human.

Inflammatory diseases caused by bacterial and viral infection include viral meningitis and bacterial meningitis, herpes encephalitis and viral meningoencephalitis, viral hepatitis, *e.g.* Hepatitis A, B, C, D, *etc.* Diseases of interest also include inflammatory response to vaccination, particularly rabies vaccine, varicella zoster vaccine, measles vaccine, *etc.*

In vitro transduction of cells with the subject DRV provides a means for screening drugs that affect the release of cytokines, and that antagonize or synergize with down-regulatory cytokines. The subject DRV can also be used in research to elucidate the mechanisms whereby down-regulatory and pro-inflammatory cytokines interact in the immune response.

The subject DRV may be prepared as formulations at a pharmacologically effective dose in pharmaceutically acceptable media, for example normal saline, PBS, *etc.* The additives may include bactericidal agents, stabilizers, buffers, or the like. The DRV may be administered as a cocktail, or as a single agent. The formulation of DRV will vary depending on whether the administration is of cells or retrovirus.

Various methods for administration may be employed. The DRV formulation may be injected intravascularly, subcutaneously, peritoneally, at the site of disease, *etc.* The dosage of the therapeutic formulation will vary widely, depending upon the nature of the disease, the frequency of administration, the manner of administration, the clearance of the agent from the host, and the like. The dose may be administered as infrequently as weekly or biweekly, or

fractionated into smaller doses and administered daily, semi-weekly, *etc.* to maintain an effective dosage level.

The formulation will be administered at a dosage sufficient to inhibit inflammation. The determination of dosage will vary with the condition that is being treated. Useful measures of inflammatory activity are the release of proinflammatory cytokines, *e.g.* IL-2, IFNg, TNFa; enhanced populations of activated T cells at disease associated sites, *e.g.* islets of Langerhans in diabetes, spinal cord in MS, *etc.*; and other measures of T cell activity as known in the art.

Mammalian species susceptible to inflammatory conditions include canines and felines; equines; bovines; ovines; *etc.* and primates, particularly humans. Animal models, particularly small mammals, *e.g.* murine, lagomorpha, *etc.* are of interest for experimental investigations.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

I. Gene Transfer of Down-Regulatory Cytokines

Genes encoding the down-regulatory cytokines interleukin 4 (IL-4), transforming growth factor (TGF-b) and interleukin 10 (IL-10), are transferred into T cells associated with insulin dependent diabetes mellitus (IDDM) in the non-obese diabetic (NOD) mouse. Expression vectors comprising the genes that encode these cytokines individually or in combination are transferred into NOD T cell clones for adoptive transfer.

A. Isolation of Disease Associated Cells

i. Isolation of T cells associated with Rheumatoid Arthritis

A set of collagen specific I-Aq restricted murine T cell clones have been isolated and characterized (Dallman and Fathman (1985) J. Immunol. 135:1113-1118). These three clones have been demonstrated to secrete cytokines consistent with the Th1 phenotype and have been shown to potentiate destructive synoviatis in adoptive transfer into young DBA/1 mice. These T cell clones are transduced with retrovirus as described herein. After retroviral-mediated gene transfer, the T cell clones are analyzed for appropriate expression of the relevant cytokine(s), both by PCR techniques and assaying for the production of the cytokine by ELISA.

ii. Isolation of T cells associated with EAE

Transgenic mice expressing a rearranged TCR containing the Va2.3 and Vb8.2 genes are described in Goverman *et al.* (1993) Cell **72**:551. This TCR is specific for the encephalitogenic myelin basic protein residues AC1-11, and was isolated from CD4+ MHC class II restricted T cell clones from the mouse strain B10.PL. The TCR transgenic mice express this receptor on the majority of their peripheral T cells. Cells were cultured by growing the transgene positive T cells in the presence of myelin basic protein AC1-11 and IL-2 or IL-4. The cells are characterized as TH1 or TH2, based on the pattern of expression of cytokines, as measured by PCR and ELISA.

iii. Isolation of NOD T cells that recognize novel autoantigens involved in early events of IDDM.

CD4+ T cell clones that were specifically reactive with islet cell extracts were isolated from young NOD mice (Gelber *et al.* (1994) Diabetes, 43:33 39). These T cell

clones and resultant hybridomas react with extracts of β cells. Fractionation of the β cell extracts demonstrated that these T cell clones recognized several different β cell specific autoantigens but none of them recognized previously reported putative autoantigens including GAD65, GAD67, HSP65, insulin, ICA69, carboxypeptidase H or perforin. These NOD T cell clones proliferated in response to similar extracts of human islets, showing a response to shared antigenic determinants between human and mouse cells (Figure 5). Additionally, these three T cell clones were shown to accelerate destructive insulitis and result in IDDM following adoptive transfer into 3 week old NOD mice, demonstrating that such T cells homed to the islets in NOD mice.

The adoptive transfer of splenocytes from diabetic NOD mice to NOD/*scid* recipients results in diabetes (Rohane *et al.* (1995) Diabetes 44:550-554). This model was employed to test the effect of cotransfer of selected T cell subsets from both young and older NOD mice into NOD/*scid* recipients. The CD4+ subset significantly delayed the onset of diabetes in splenocyte co-transfers.

To further characterize the "down-regulatory" $CD4^+$ cells, $CD4^+$ subsets were examined based on the expression of the CD45 cell surface determinant. The population was divided into a CD45RB low population, representing approximately 20 to 30% of peripheral lymphocytes ("memory" T cells) and a CD45RB high population representing about 60 to 70% of the peripheral lymphocyte CD4+ subset ("naive" T cells).

The CD45RB low subset of $CD4^+$ cells from young non-diabetic mice provided protection from diabetes in a co-transfer with splenocytes from overtly diabetic NOD mice. Similar transfers of CD45RB low cells from diabetic NOD mice had no protective effects in co-transfer.

Cytokine analyses were performed on the CD4⁺CD45RB low and high subsets, following anti-CD3 activation. 3 x 10^5 splenocytes from diabetic (n=6), young (8-12 week old, n=12), or older (20-24 week, n=8) NOD or BALB/c (13-14 weeks, n=4) mice were stimulated with anti-CD3 antibodies for 40-48 hours with APCs (6 x 10^5 3000 rad irradiated CD4 depleted splenocytes). Supernatants were analysed by ELISA. The data is shown in Figure 6. CD45RB low cells from diabetic mice showed a significantly higher ratio of interferon g to IL 4 release, when compared to CD45RB low cells from non-diabetic mice.

There was a marked difference in the cytokine profile when CD45RB low CD4⁺ cells were taken from age matched non-diabetic old NOD mice and compared to the same set of cells from age matched diabetic NOD mice. The CD45RB low subset from old non-diabetic NOD mice had a much lower interferon g to IL 4 ratio (similar to that seen in the non-diabetogenic CD45RB low CD4⁺ population from young NOD mice). The same population from the diabetic mice had a very high interferon g to IL 4 ratio, suggesting that Th 1 cells predominated in the CD45RB low CD4⁺ population from diabetic mice, but that Th 2 like cells predominated in the same population from age matched non diabetic mice.

CD45RB low CD4⁺ splenocytes (5 x 10^5) from diabetic (n=4) or older non-diabetic (20-24 weeks, n=4) NOD mice were transferred into NOD/scid recipients together with 5 x 10^5 CD8+ splenocytes from diabetic mice. The CD45RB low cells from older, age matched non-diabetic mice did not transfer diabetes in co-transfer with CD8⁺ cells from diabetic NOD mice into NOD/*scid* whereas CD45RB low cells from diabetic mice did. The data is shown in Figure 7. These data provide evidence that down-regulatory cytokines from Th 2 like cells protect cells from destruction.

B. Construction of retroviral constructs for the constitutive or inducible expression of the cytokines IL-4, TGF and IL-10.

A rapid, stable retrovirus production system allowing large-scale stable generation of biomedically relevant levels of high titre recombinant retrovirus is used. Vectors and cell lines are used that allow rapid establishment of stable retroviral producer DNA as Epstein-Barr Virus (EBV)-based episomes within 293 cell human producer systems. Thus, large recombinant producer cell populations, and bulk retrovirus for concentration, are readily generated from small seed populations.

Retrovirus producer cell lines are based on the 293T cell line, a human embryonic kidney cell line transformed with adenovirus E1a and carrying a temperature sensitive T antigen co-selected with neomycin. This cell line is highly transfectable with either $CaPO_4$ mediated transfection, or lipid based transfection protocols. The first generation producer cell lines had defective constructs capable of producing gag-pol and envelope protein for ecotropic and amphotropic viruses. The original cells have been improved by the addition of an IRES-CD8 surface marker downstream of the reading frame of the gag-pol construct. Thus, CD8 expression is a direct reflection of the intracellular gag-pol. The stability of the producer cell population's ability to produce gag-pol can be readily monitored by flow cytometry. For both the gag-pol and envelope constructs, non-Moloney promoters were used to minimize recombination potential, and the two promoters were different, to minimize their inter-recombination potential. Two cell lines were produced, øNX-eco and øNX-ampho (øNX-a). Gag-pol was introduced with hygromycin as the co-selectable marker, and the envelope proteins were introduced with diphtheria resistance as the co-selectable marker.

Both øNX-eco and øNX-ampho have been tested for helper virus production and established as being helper virus free. Both lines are capable of carrying episomes for long term stable production of retrovirus. Both lines are readily testable by flow cytometry for stability of gag-pol and envelope expression; after one month of testing the lines were more stable than the first generation lines. Both lines can be used to transiently produce virus in a few days. Thus, these lines are fully compatible with transient, episome stable and library generation for retroviral and gene transfer experiments.

The vector termed LZRS-lacZ, shown in Figure 2, is the base vector into which down-regulatory genes are inserted. The vector is shown with the retrovirus at the top of the figure inserted into the vector which contains a puromycin resistance gene expressed from PGK origin. The down-regulatory gene region depicts an approximately 6.5 kb region that can accept DNA. In LZRS-lacZ it is lacZ. An EBV origin is depicted and contains the family of repeats, the dyad repeats, and EBNA.

Two classes of vectors are constructed. The first vector expresses the appropriate down-regulatory cytokine under the control of the retroviral promoter (a powerful, constitutively expressing promoter). The Long Terminal Repeat (LTR) of the murine retrovirus, which contains the enhancer and promoter of the retrovirus, is capable of expression in multiple hematopoietic cell lineages. Retroviruses are the vector of choice for delivery of genes to hematopoietic cells. Figure1A to 1D show basic retroviral constructs. The plasmid backbone of the vector shown in Figure 2 contains the episomal maintenance features EBNA/Ori/Puro, which allow for the rapid establishment of high titre retroviral producer lines.

The nuclear replication and retention functions of the Epstein-Barr virus have been used to maintain

retroviral vectors episomally within human-based retroviral packaging cell lines. These hybrid EBV/retroviral vectors are capable of producing helper-free recombinant retrovirus as soon as 48 hours, and for at least 30 days after transfection into 293T-base ecotropic and/or amphotropic retroviral packaging cells. Viral titers greater than 10^7 CFU/ml were obtained after puromycin selection of transfected retrovirus packaging cell lines. This episomal approach to retroviral production circumvents limitations inherent in transient and chromosomally-stable retroviral producer systems, thereby affording reproducibly rapid, large-scale, high titer retrovirus production.

EBV-based retroviral vectors have been designed that take up stable residence as episomes within human retroviral producer cells at an efficiency close to their transient transfection frequency. In human 293 cells, the rate of stable establishment of EBV-based episomes can reach 25% of the starting cell populations after $CaPO_4$-mediated transfection. To establish stable episomes, it is required that the Epstein Barr Nuclear Antigen (EBNA) binds to the EBV origin or replication and nuclear retention sequences. The episome is thereby maintained at 5 to 20 copies per cell for up to two or three months, given a puromycin-resistance gene resident and selected for on the plasmid. The prototype retrovirus vectors use lacZ as the marker for rapid, accurate determination of titer. The vector incorporates a number of important features. A more efficient retroviral LTR has been designed into the vector, based on the MFG retroviral backbone of Mulligan *et al.* (*supra.*) The vector has been designed to accept retroviral backbones from a number of different systems, facilitating the movement of previously designed retroviruses based on old systems, to this more efficient system. A suitable polylinker region has also been incorporated. In tests of this vector it was determined

that retroviral titers as high as 10^7 can be readily generated. These titers are achieved within 5 to 10 days after initiation of the culture, and are higher than any reported system.

pBabePuro has been previously described (Pear *et al., supra.*). It contains LTR and y packaging sequences from the Moloney Murine Leukemia Virus (Mo-MLV). The puromycin resistance gene is present within the retroviral backbone and under the transcriptional control of the SV-40 early promoter.

pBabePuro (ΔSV40) is a derivative of pBabePuro, in which the SV40 early promoter has been removed by digestion with SalI and HindIII, followed by an end-filling reaction with Klenow fragment and religation of the blunted vector.

pBabePuro(ΔSV40)A was constructed by insertion of an adapter (5'-BglII-HindIII-XhoI-EcoRI-3') into a unique NotI site present within the backbone of pBabePuro(ΔSV40); the NotI site was not reconstructed.

p220-2 contains the EBV EBNA-1 gene, EBV Orip cis elements, and the hygromycin resistance gene in a pBR322 backbone. Proximal to the Orip sequences are mRNA termination sequences from the herpes simplex virus type I thymidine kinase gene. Additionally, p220.2 contains termination sequences from the HSV type-1 thymidine kinase gene. These sequences are positioned to prevent opposing transcriptional run-through into the EBV Orip cis elements which, if left unchecked, can suppress Orip mediated replication.

PGKPuro was created by ligating the puromycin resistance gene and SV40 polyadenylation sequences (PstI-BamHI fragment) from the plasmid pPur into the PstI and BamHI sites of the plasmid PGKNeo-Sut-1. The resulting vector contains the SV40 polyadenylation sequences and the puromycin resistance gene driven by the Phosphoglycerol kinase-1 promoter (PGK-1).

pBab Puro-LacZ is a derivative of pBabePuro in which the LacZ gene, obtained from the plasmid AVRZ (DraI fragment) has been blunt end ligated into the SnaBI polylinker site of pBabePuro.

pBabeM is a retroviral vector constructed using the 5' LTR and y packaging sequences of the MFG vector (PstI-BamHI fragment) and the LacZ gene and 3' LTR of pBabePuro-LacZ (PstI-BamHI fragment). Polylinker sequences that flank the LacZ gene of pBabeM were constructed using a DNA synthesizer and standard techniques.

pBabePuro^{220} was created using sequences from pBabePuro(ΔSV40)A and p220.2. The EcoRI-BamHI fragment of p220.2, which contains the EBV Orip elements and the EBNA-1 gene, was inserted into the BglII and EcoRI adapter sites of pBabePuro(ΔSV40)A.

pBabePuro^{220}(A) was constructed by insertion of a 67 base pair adapter (5'-NaeI-BamHI-HindIII-NotI-SalI-AseI-NruI-PmlI-BglII-3") into the EcoRI site of pBabePuro^{220}.

pREPP(A) was created by inserting the EcoRI-BamHI fragment of PGK-Puro, which contains the PGK-1 promoter, the puromycin resistance gene and SV40 polyadenylation sequences, into the EcoRI and BglII sites of pBabePuro^{220}(A).

LZRS-LacZ(A) was created using sequences derived from pREPP(A) and pBabeM. BspHI digestion of both pREPP(A) and pBabeM, followed by ligation of the appropriate fragments, yielded LZRS-LacZ(A).

LZRSp$^{BMN\text{-}LacZ}$ is identical to LZRS0LacZ(A) with the following modifications: a previously unrecognized ATG start site present 5' to the pBabeM viral polylinker was blunted/destroyed. The restriction sites remaining in the backbone polylinker (NaeI-EcoRI) were destroyed.

pBabePuro-LacZ220 was constructed by ligating the appropriate BspHI fragments from pBabePuro-LacZ and pREPP(A). This vector contains the SV40 promoter, which can function as a lytic origin of replication, provided

Large T antigen is supplied in trans. 293T-based retroviral packaging lines stably express a temperature sensitive version of SV40 Large T antigen, and therefore transient incubations at the permissive temperature may induce lytic replication of pBabePuro-LacZ220 to high copy numbers. Such increases in vector copy number (up to 10,000 copies/cell) may increase the total yield of infectious virus produced from this episomal vector.

The DNA sequence for the coding regions of the cytokines IL 4, TGFb and IL 10 are accessible through Genbank, as previously referenced. Inserts in expression vectors driven by the murine insulin promoter have been subcloned into pUC 18 by excision of the gene at a unique Xba I site 5' of the coding region and a flanking 3' Aat II site and inserted into the corresponding sites in pUC18.

E. coli transformants were screened both by PCR, using primers unique to each coding sequence, and by restriction digest analysis. The cytokine genes were then subcloned from the pUC plasmids with a 5' Eco RI and a 3' SspI site (blunt end) into pBABE vectors (Morgenstern and Land (1993) N.A.R.) that had first been digested at the unique Sal I cloning site, blunted ended by treatment with Klenow, purified and then cleaved at the Eco RI cloning site. Transformed colonies were analyzed as above.

The genes are subcloned into the retroviral vectors. Individual retroviral backbones expressing appropriate cDNAs are transferred into the episomal backbone LZRS. The lacZ encoding DNA of LZRS is replaced by the retroviral backbone of the cDNA encoding virus in pBabeM, an MFG derivative having significantly higher infection efficiency for T cells. They are incorporated into the episomal backbone vector of LZRS to allow for long term, high volume production of retroviruses.

The second class of vectors utilize an inducible expression system under the control of the NF-AT (Nuclear

Factor of Activated T cells) minimal promoter. This vector provides limited expression of the introduced down-regulatory cytokine gene, but only after antigen stimulation. The NF-AT regulatory system maintains exquisite control over gene expression, and is rapidly induced following T cell stimulation. The vector is shown in Figure 1C and 1D. It utilizes the self inactivating technology of 3' U3 deletion in combination with the episomal maintenance system.

NF-AT inducible retroviruses are designed incorporating the Self Inactivating (SIN) feature of a 3' LTR enhancer/promoter retroviral deletion mutant (Yu *et al.* (1986) P.N.A.S. 83:3194-3198). The U3 region of the 3' LTR is used during retroviral reverse transcription for creation of the next generation's 5' LTR enhancer promoter region. Removal of the 3' LTR promoter and enhancer elements from a recombinant retrovirus leads to the production of retroviruses that are transcriptionally defective after integration in the target cell. Internal incorporation of a promoter conferring constitutive, stage specific, or inducible expression then allows for non-retroviral control over insert expression.

The NF-AT minimal regulatory region is incorporated as the internal inducible promoter in the SIN vectors. This promoter configuration (3 tandem NF-AT sites immediately upstream of the interleukin 2 minimal promoter) provides T cell specific inducible expression of cloned inserts. These vectors are incorporated into the LZRS episomal backbone for production of high titer stable packaging lines (see Figure 2). In the absence of T cell induction the promoter is completely shut down. T cell activation leads to the rapid induction of gene expression from the introduced construct (Fiering *et al.* (1990) Genes and Dev. 4:1823-1834).

The NF-AT inducible promoter is excised from NFAT lacZ (Fiering, *supra*.) and inserted into the SIN vector pMGFPro as shown in Fig. 5. PMGFPro is a self inactivating derivative of MFG lacZ. The enhancer/promoter region in the 3' LTR of this vector has been deleted from the first Pvu II site to E the Sac I site, thus deactivating the LTR of the next generation. These vectors are incorporated into the LZRS episomal backbone for production of high titer stable packaging lines.

IRES constructs are used to track expression of cytokines and marker genes. Retroviruses containing both a cytokine gene and a fused IRES-reporter gene (green fluorescent protein, GFP, or lacZ) are expressed dually from the retroviral LTR. In this manner, in histological stains for lacZ or GFP in tissue sections the level of expression of lacZ GFP is representative of the expression of the included cytokine.

C. Transduction of EAE inducing hybridomas using retroviral vectors.

The genes encoding IL-4; IL-10 and LacZ (as a negative control) were inserted into the LZRS retroviral vectors as described above. The encephalitogenic murine T cell hybridoma G1.15H was transduced with each of the three retroviral constructs. The resulting transduced cells were analyzed and shown to express the appropriate cytokine or LacZ gene product. The IL-4 secreting hybridoma produced 40 ng/ml of IL-4, and the IL-10 secreting hybridoma produced 2-4 ng/ml of IL-10.

D. Adoptive transfer of transduced T cell clones into EAE mice.

Experimental autoimmune encephalitis was induced in four groups of 4 mice each (PL/J x SJ/L F_1). EAE was actively induced by subcutaneous immunization with myelin

basic protein (MBP) in CFA plus 0.2 mg *Mycobacterium nuberculoris* H37RA (Difco Laboratories, Inc., Detroit, MI). EAE was scored clinically as follows: no neurological signs = grade 0, weak tail = grade 1, wobbly walk or limb paresis = grade 2, limb paralysis = grade 3, inability to move = grade 4, death = grade 5. Easy access to food and water was provided and animals with grade 3 or 4 received daily parenteral fluid.

Ten days after injection of MBP, the groups were treated as follows: A. untreated; B. injected with 10^6 LZRS lacZ transduced G1.15H cells; C. injected with 10^6 LZRS IL-4 transduced G1.15H cells; D. injected with 10^6 LZRS IL-10 transduced G1.15H cells. The animals were then monitored for development of disease. The results are shown in Figure 11. The data show that the group of animals treated with the LZRS IL-4 transduced hybridoma were protected from an otherwise severe inflammatory disease.

E. Adoptive transfer of transduced T cell clones into mice.

Insulitis in NOD mice is treated by adoptive transfer of NOD T cell clones transduced with genes expressing down-regulatory cytokines. The T cell clones are transduced with the retroviral vectors described above, that lead to cytokine expression. The transduced cytokine secreting T cells are used in adoptive transfer along with the mock transduced and Lac Z transduced control T cell lines into both conventional NOD mice and into Rag 2 knockout NOD mice.

Tissue localization of the T cells in the islets of Langerhans is traced by the Lac Z construct (histopathology), by *in situ* hybridization studies for the cytokine, and FACS analysis using single cell suspensions of the islets of the adoptively transferred NOD mice for FITC labeled cells. The adoptively transferred cells are

tracked into the appropriate sites of potential tissue destruction.

Co-adoptive transfer of graded numbers of the T cell clones along with diabetogenic cells from conventional NOD mice is used in order to determine whether the T cells expressing down regulatory cytokines block the rapid transfer of diabetes in the Rag 2 knockout/NOD mouse. An adoptive transfer model is also used in which conventional NOD mice of different ages are given graded numbers of the transduced T cell lines. The degree of islet infiltration (insulitis) is followed in mice that receive the mock transduced cells, as well as the cytokine secreting transduced T cells.

A construct for inducible cytokine expression using NF-AT promotion is also used. The cytokine is expressed following appropriate antigen recognition by the transduced T cell clone. T cell clones transduced with the NF-AT promoter driving expression of the cytokine are transferred along with T cells from diabetic or prediabetic NOD mice into the NOD/Rag 2 knockout mice. The T cell clones bearing the inducible cytokine gene(s) are also transferred into NOD mice of various ages.

The transduced T cell clones identified as expressing the cytokines of interest (IL 4, TGFb, IL 10), are adoptively transferred into NOD/Rag-2 knockout and NOD mice. The T cell clones are additionally stained with FITC to follow their tissue localization. The effects of transfer is monitored at the following time points:

1. NOD mice of 3-4 weeks (coincident with the first visible infiltrates)

2. NOD mice of 6-8 weeks (when insulitis is well established)

3. NOD mice at about 100 days (shortly before overt hyperglycemia)

4. NOD mice one week after onset of overt diabetes.

Islet infiltrating T cells of NOD mice > 40 days old rapidly transfer IDDM to NOD/scid mice (< 30 days). Cells taken from the periphery of 40 day old NOD mice have a very delayed time in transfer of disease to NOD/scid mice, requiring approximately 100 days to develop disease. Peripheral lymphocytes and islet infiltrating lymphocytes from NOD mice at 40 and 80 days of age are transferred into NOD/Rag-2 recipients. Kinetics of the disease is followed by the histologic changes of insulitis and following mice for overt hyperglycemia. Groups of mice are compared that have either received mock transduced T cell clones or clones expressing down-regulatory cytokines in co-transfer with the diabetogenic T cells.

II. Gene Transfer of Negative Dominant Molecules

A. Construction of retroviruses Expressing Dominant Negative Signalling Molecules

Retroviruses at high titre are constructed with genes encoding dominant negative inhibitors of NFkB. The first class of retroviruses is constructed to block intracellular signaling leading to proinflammatory cytokine expression using dominant negative polypeptides active in the NF-kB signaling cascade. Inhibition of NF-kB activity has been shown to lead to blocking of proinflammatory events.

NFkB p50 dominant negative retroviruses: The dominant negative p50 NF-kB genes are deleted for their DNA-binding region, but retain their dimerization motif. Dimerization between NF-kB monomers is essential for their ability to bind DNA to activate transcription. Dominant negative molecules thereby block NF-kB dependent gene expression. NF-kB p50 can promiscuously heterodimerize with all known NF-kB Rel monomers. Delivery of a dominant negative p50, deleted for its DNA-binding domain, will

effectively shut down all NF-kB mediated signaling in cells.

Retroviruses capable of high level expression of NF-kB p50 are described in Nolan *et al.* (1993) Mol. Cell. Bio. 6:3557-3566. A mutant NF-kB p50 molecule deleted for its DNA-binding domain and capable of expression in mammalian cells was made and expressed via retroviral delivery to mammalian cells. NF-kB p50 mutant deleted for its DNA binding domain (residues 33 through 54) was expressed in NIH 3T3 cells. Retroviral vectors were used to generate retroviral supernatants. The supernatants were infected into NIH 3T3 cells previously plated onto coverslips in 24 well plates. Two days after infection, cells on coverslips were fixed and stained for p50 expression using a polyclonal anti-NF-kB p50 as described in Nolan *et al.*, *supra*.

The retroviral vector LZRS-p50-DBD (DNA-Binding Domain) is created by inserting the appropriate retroviral fragments into the long-term episomal production vector LZRS. The NF-kB and IkB retroviral constructs are shown in Figures 8A to 8C. This vector is used to establish long term episomal production of p50 DBD, a gene deleted for the DNA binding domain of NF-kB p50 Rel region. An IRES neomycin fusion construct is added to the vector to allow for single promoter expression of both the NF-kB p50 DBD gene and the neomycin phosphotransferase gene, which will confer G418 resistance upon cells that have stably integrated and expressed the retrovirus. Stable retroviral producer lines producing a retrovirus with a dominant negative p50 protein are established after less than one week after transfection and maintenance in puromycin.

Target T cell clones (diabetogenic $CD4^+$ T cells) are appropriately activated with antigen by antigen presenting cells (stimulation of 3×10^5 responder T cells with 0.5×10^6 irradiated (2,000R) spleen antigen presenting cells).

At 48 hours target cells are placed in the in the presence of 10 ml of retroviral supernatant from BOSC23 cells with an established p50-DBD episome. Retroviral supernatant is removed after 6 hours and the final stages of infection are allowed to proceed for 3 days, after which antigen presenting cells and T responder cells are removed and placed in a fresh tissue culture vessel.

Diabetogenic CD4+ T responder cells are then stimulated as above with irradiated APC in the presence of 1 mg/ml G418 to select for T responder cells that express the retroviral inserts (both p50 DBD and neo). The cells are expanded by conventional methods. 5 x 10^6 cells are lysed and protein prepared for Western analysis of p50-DBD expression using anti-p50 polyclonal antibody as primary. As control lysates, protein is prepared from NIH 3T3 cells, NIH 3T3 cells infected with p50-DBD, T responder cells infected with control LZRS-IRES neo (lacking the p50-DBD gene), and non-infected T cells.

IkB dominant negative retroviruses: Dominant negative forms of IkB are an alternative to dominant negative NF-kB p50. IkB is the specific regulator of NF-kB p50:p65 heterodimers. It is a cytoplasmic protein associated with NF-kB prior to its activation. Signaling events both at the cell membrane and internally lead to a series of upstream events that integrate in the phosphorylation and proteosome degradation of IkB. Two regions of IkB are essential for the necessary degradation process. One region included the carboxyl terminal non-ankyrin domain of IkB and the second has been localized to two phospho-serine residues in the amino terminus non-ankyrin region (positions 32 and 36). Figure 8B illustrates IkB protein domain regions. The five recognized ankyrin regions (35 amino acid repeat structure predicted to form a helix bundle structure) are designated

as open ovals. Denoted are the two phosphoserine residues at positions 32 and 36 implicated in induced activation of IkB. Also indicated is a 39 amino acid region at the carboxyl terminus of IkB that is thought to be involved in the basal phosphorylation of IkB necessary for induced activation. Both regions, the 32/36 and the 39 amino acid carboxyl region, when mutated, cause IkB to act as a dominant negative in T cells.

Retroviruses expressing these IkB mutants are expressed at high levels in 70Z/3 B cells, shown in Figure 9. A mutation at residue 32, (serine glycine), designated pBabePuroIkB, and a deletion mutant lacking the carboxyl-terminal 39 residues, designated pBabePuro IkB, were derived in these vectors by standard methods. Virus was created by the method described in Pear *et al.* (1993) P.N.A.S. **90**:8392-8396 and supernatant from these transfections, containing retrovirus, was used to infect 70Z/3 cells. Transduced cells were selected in 1ug/ml puromycin to derive a pure population of retrovirally transduced cells. Lysates were prepared from these cells and used in a Western analysis with anti-human IkB antibody. The figure shows lane 0: molecular weight markers; lanes 1-4 are stimulated 40 ng/ml PMA + 2 uM ionomycin for 60', 15', 5', 0' with mutant IkB containing a serine to glycine mutation at position 32; lanes 5-8 are the same stimulation conditions and timing using a retrovirus with wild-type IkB-; lanes 9-12 are the same stimulation conditions with uninfected cells. High-level expression of the mutant IkB isoform containing the residue 32 mutation is seen. Other mutations, including double and deletion, showed the same effect. Note that the 60' time point in the mutant is still strongly expressing.

These individual IkB mutants are transferred into the episomal production vector LZRS (generically termed LZRS-I IkB /mut). Long-term cultures of retrovirus

producing cells is established and used to infect T cell clones. The cells are expanded by conventional methods and 5 x 10^6 cells are lysed and protein prepared for Western analysis of LZRS-IkB /mut expression using anti-IkB polyclonal antibody as primary antibody (Santa Cruz Biologics). Protein prepared from NIH3T3 cells, NIH 3T3 cells infected with LZRS-IkB/mut, responder cells infected with control LZRS-IRESneo (lacking a IkB/mut gene), and non-infected T cells is used to verify-IkB/mut expression.

B. Effect of Dominant Negative Genes on NF-kB Specific Signaling.

The effect of the dominant negative genes described above on the secretion of inflammatory cytokines (g interferon) following mitogen or specific antigen activation is determined. After verification of dominant negative NF-kB and IkB expression (p50 -DBD and IkB/mut, respectively), the virally-infected CD4+ T cell population is tested for specific down-regulation of NF-kB activity on reporter constructs as compared to Rous Sarcoma Virus promoter driving luciferase (RSV-luc), a promoter known to be unaffected by NF-kB signaling pathways (see Table 1).

Table 1

	Stimulation Regime		
retrovirus construct	mock-activated	CD3/CD28 co-activated	g-interferon
LZRd IRES neo	1. NF-kB-luc 2. RSV-luc	1. NF-kB-luc 2. RSV-luc	1. NF-kB-luc 2. RSV-luc
LZRS-p50D-DBD	1. NF-kB-luc 2. RSV-luc	1. NF-kB-luc 2. RSV-luc	1. NF-kB-luc 2. RSV-luc
LZRS IkB-a/D39	1. NF-kB-luc 2. RSV-luc	1. NF-kB-luc 2. RSV-luc	1. NF-kB-luc 2. RSV-luc
LZRS IkB-a/32-36	1. NF-kB-luc 2. RSV-luc	1. NF-kB-luc 2. RSV-luc	1. NF-kB-luc 2. RSV-luc

500 ng of the appropriate reporter construct, see Table 1 above, is transfected by the DEAE-Dextran method into $CD4^+$ T cells infected with the indicated retroviral constructs (1×10^6 per sample) and treated as per Table 1 (cells are either mock-activated or CD3/CD28 co-activated to mimic specific mitogen). Mock-activated cells are activated with anti-IgM of the same allotype(s) as the CD3/CD28 coactivators. TNF activations (50 ng/ml) are done on cells four and five days after stimulation with CD3/CD28. This will allow for Rel induced by mitogenic stimulation to subside, such that interferon-g activation can be effectively measured, while ensuring that cells are still in an active cell cycle phase conducive to proinflammatory signaling. CD3/CD28 co-activated cells are transfected at days two and three after activation. Luciferase activity is measured by standard assays.

Infected T cell clones are studied for down-regulation of proinflammatory cytokine production. Their capacity to be induced by specific mitogens to express g interferon is a measure of proinflammatory signaling processes. Resting $CD4^+$ cells (1×10^6) are stimulated as

above with IgM for mock-activation, or with CD3/CD28 coactivation. Total RNA is prepared from stimulated cells. g interferon transcription is measured using Polymerase Chain Reaction (PCR) primers corresponding to the murine cDNA for g interferon. The identity of PCR-recognizable band is verified by Southern blot using the g interferon cDNA. PCR reactions are run for 10, 20, and 30 cycles to ensure that quantitation of PCR product is a reflection of RNA levels and that the PCR has not run to completion. Also, ELISA for g interferon is done to verify secretion is down-modulated as well.

C. Construction of Retrovirus Expressing Antibody Genes

One cytokine, TNF, is predominantly involved in the early pathogenic lesions of insulitis in NOD mice. Retroviral vectors are constructed containing a gene encoding a single chain anti-TNF antibody that can be secreted by CD4 cells following a) transduction *in vitro* and b) targeted gene transfer to OX-40$^+$ CD4$^+$ cells in lesions of insulitis.

Anti TNF- single chain variable fragment: Heavy and light chain variable regions of antibodies can be expressed as a single linear polypeptide. Introduction of a glycine-serine spacer between a fused heavy and light chain allows assembly of a functional variable region "fragment" that retains specificity and affinity for the original target antigen. In this manner, single-chain antibody variable fragments (scFv) are engineered onto various polypeptide backbones and secreted from cells.

T cells are engineered to secrete single chain variable regions with neutralizing activity against TNF. The antibody is one that has broad experimental usage as a neutralizing antibody with high affinity for TNF, a hamster anti murine TNF clone termed TN3.19.2 (Williams *et al.* (1992) P.N.A.S. **89**:9784-9788).

Single chain antibody against TNF is engineered according to the secretion scheme of Jost *et al.* (1994) J.B.C. **269**:26267-73. Specifically, mRNA is prepared from the monoclonal cell line TN3.19.2, which produces hamster IgG1 antibody to murine TNF b. Primers corresponding to hamster IgG1 from heavy chain variable regions are used to reverse-transcribe mRNA from TN3.19.2. Similarly, hamster light chain constant region primer is used to reverse transcribe the light chain. Clones are detected using a labeled primer upstream of the IgG1 primer as probe. The sequences is verified for complete open reading frames. A Southern blot is performed with verified clones and a constant region probe to ensure that only one variable region set exists in the monoclonal cell line.

The heavy and light chains are inserted into a single chain variable fragment vector using appropriately engineered sites. The new vector is termed pscFv-TN3. In this vector a glycine-serine spacer region (Gly 4 Ser) linker is inserted between the carboxyl terminus of the heavy chain variable region and the amino terminus of the kappa light chain variable region. The (Gly 4 Ser) linker separates the heavy and light chains encoded on a single polypeptide backbone. The construct is inserted into the retrovirus vector LZRS to fuse the heavy chain region of a secreted form of murine IgG1 to the scFv-TN3 variable region. The vector is termed LZRS-TN3IgG. In this vector the antibody region to TNF b is secreted from cells on a secreted IgG1 polypeptide backbone.

The vector is tested in the retrovirus production system for expression of the TN3-IgG1 epitopes and TNF specificity. Supernatants from BOSC23 cells transfected with the LZRS-TN3IgG vector are tested for their neutralizing capacity against known amounts of TNF in solution. Assays for neutralizing specific activity are done. Affinity of the TN3IgG1 fusion protein for TNF by

competition is quantitated with known amounts of TN3.19.2.

Retroviruses from an established episomal producer line are used to infect NIH3T3 cells and diabetogenic T cells clones. Antibody production and its neutralizing capacity as expressed from NIH 3T3 cells (positive controls for infection and expression) and T cell clones is measured. TH-1 T cell clones are infected to express TN3IgG1.

D. Adoptive transfer of T cells transduced with down-regulatory retroviral constructs.

Retroviral transduction is accomplished as described above, with vectors containing the dominant-negative NF-kB, IkB, or anti-TNF antibody construct.

Adoptive transfer of $CD8^+$ T cells taken from overtly diabetic NOD mice into NOD/*scid* mice does not allow the transfer of diabetes within at least two months following transfer. When $CD4^+$ T cells are adoptively transferred along with the $CD8^+$ T cells, diabetes can be demonstrated in the same recipients within 3 weeks. Diabetogenic $CD4^+$ T cell clones are add-mixed with $CD8^+$ T cells from overtly diabetic NOD mice and transferred into NOD/RAG-2 knockout mice to demonstrate transfer of diabetes.

The adoptive transfer of $CD8^+$ T cells from frankly diabetic NOD mice into NOD/RAG-2 mice is tested with and without mock transduced Th-1 diabetogenic CD4+ T cell clones or with the CD4+ Th-1 diabetogenic T cell clones carrying genes encoding dominant-negative NF-kB, IkB.

The CD4+ T cell clones carrying dominant-negative NF-kB, IkB genes are used in studies of transfer into young NOD mice to assay for the ability to accelerate diabetes. These studies are controlled by using the mock transduced T cell clone appropriate to the studies.

T cell clones transduced with genes encoding the single chain anti-TNF antibody are used in adoptive transfer. These clones are transferred into NOD/RAG-2 knockout mice along with diabetogenic T cells from NOD mice. The clones are also transferred into NOD mice of various ages to determine the effect of anti-TNF at the site of the insulitis lesion. Adoptive transfer is performed in the same four groups of mice as previously described:

1. NOD mice of 3-4 weeks (coincident with the first visible infiltrates)

2. NOD mice of 6-8 weeks (when insulitis is well established)

3. NOD mice at about 100 days (shortly before overt hyperglycemia)

4. NOD mice one week after onset of overt diabetes.

The second analysis of anti-TNF antibodies is done by adoptive cotransfer of the CD4+ Th-1 diabetogenic T cell clones transduced with a gene encoding a single chain anti-TNF antibody along with diabetogenic T cells from various sources transferred into NOD/RAG-2 knockout mice. These studies analyze whether secretion of anti-TNF antibody blocks the rapid transfer of diabetes.

V. Construction of OX-40 Targeted Retrovirus

A retroviral packaging system containing a disease down-regulating cytokine is constructed using an OX 40 ligand to target activated cells *in vivo*. The delivery system takes advantage of the selective expression of OX 40 on antigen activated T cells present in the lesion. The OX 40 marker is characteristic of $CD4^*$ T cells at sites of inflammation in autoimmune diseases. By incorporating the ligand for OX 40 in the coat protein of the retrovirus, genes are delivered specifically to activated $CD4^*$ T cells.

Retroviral producer cell lines expressing both ecotropic receptor and two classes of retargeting vectors are made. One will incorporate the OX-40 ligand as the retargeting module in the ecotropic envelope protein. The second will utilize a single chain antibody variable fragment directed against the OX-40 molecule as the retargeting module.

Figure 10 depicts the bipartite retroviral envelope protein in the lipid bayer of a mature retroviral particle. The specificity determinant resides in the extraviral loop section. The transmembrane section is a proteolytically cleaved fragment that contains the fusion function for the virus to enter the cell. The tropism of the various envelope polypeptides for mammalian cells is shown. Also depicted is a retargeting scheme bringing an OX-40 ligand fusion into the ecotropic envelope loop region. It is co-expressed on viruses with wild type ecotropic receptor, which provide in trans those functions destroyed by OX-40 ligand function.

For infection of murine cells, the xenotropic envelope will be adapted to allow retargeted, specific infection of murine cell subsets. The xenotropic retroviral envelope, related to both the ecotropic and the amphotropic envelopes, will allow retroviruses to efficiently infect many mammalian cells type, but not murine cells. The xenotropic envelope protein allows infection of murine cells in an analogous manner to the use of the ecotropic engineering.

A. Incorporation into the viral coat an OX 40 specific, single chain antibody variable fragment (scFv) derived from a monoclonal antibody.

The heavy and light chain variable regions of antibodies can be expressed as a single linear polypeptide as described above. The antigen specificity of the single

chain variable region provides the retrovirus with the capacity to selectively infect OX 40 positive, activated $CD4^+$ T cells.

The heavy and light chain variable regions from a monoclonal hybridoma line that produces antibody against the OX 40 antigen is cloned as follows. mRNA is prepared from the monoclonal cell line L106, which produces murine IgG1 antibody to the human OX 40 surface antigen. Primers corresponding to all IgGs except IgG3 and degenerate primers with the sequence to heavy chain variable regions is used to amplify randomly primed reverse transcribed mRNA as described in Coloma *et al.* (1992) J. Imm. Meth. 152:89-104. Similarly, k light chain constant region primer and degenerate primers are used to PCR clone light chain. These are cloned into sequencing vectors by TA cloning and the sequences verified for complete open reading frames. A Southern blot is performed using the cloned genes as a probe to ensure that only one variable region set exists in the monoclonal L106.

The leader region and N terminal residues of the murine ecotropic envelope protein are shown with an engineered insert at the Bst EII site in Figure 3. The cloned heavy and light chains are inserted into the single chain variable fragment vector using appropriately engineered sites. A glycine serine spacer region separates the heavy and light chains encoded on a single polypeptide backbone. The construct is inserted into the fusion envelope vector at the Xho I and Bst EII sites as per Figure 3A.

A xenotropic retargeting system for infection of mouse cells based on the AKR derived xenotropic envelope protein is constructed as follows. This retroviral envelope protein is incapable of infecting murine cells, but has analogous fusion regions. Specificity for murine OX 40 is introduced with the murine OX 40 ligand. The

murine ecotropic and xenotropic envelopes show a very high degree of homology, except in areas thought to be important for determining specific ligand binding. The sequence of the xenotropic region is shown in Figure3D. The sequence is modified by insertion of an Xho I and Not I site by standard PCR mutagenesis methods. The leader region and N terminal residues of the murine xenotropic envelope protein are shown with an engineered insert at the Not I site. Constructed envelope fusion vectors are transfected into 293 cells, and the cells subsequently stained with the monoclonal antibody 83A25 against determinants on MMULV envelope protein. FACS screening tests for expression of the mature envelope protein. Pseudotyped envelope is used to create retrovirus to test infection of OX 40 expressing cells.

B. Incorporation of OX 40 Ligand into Retroviral Envelope

The retroviral constructs are made by inserting DNA encoding the ligand region of human and murine OX 40 ligand into ecotropic and xenotropic retroviral envelope genes. Using these envelopes, cell lines are created based on ecotropic or xenotropic retrovirus producer systems. Episomal vectors are established that are capable of sustaining long term recombinant retroviral vector expression. Supernatants from the newly established producer lines are used to infect target cells.

Cell lines engineered to express human OX 40 receptor are infected *in vitro*. The OX 40 receptor is inserted into the retroviral vector pWZL neo by PCR techniques. The Internal Ribosome Entry Site (IRES) allows neomycin to be under the control of the LTR coordinately with OX 40 expression. Drug selection for G418 resistance conferred by expression of neomycin phosphotransferase protein assures expression of the OX 40 receptor on all cells. HeLa cells and Namalwa B cells are engineered to

express human OX 40 by infection with the vector, and selection in G418. Control cells are infected with a similar vector expressing the CD8 gene in place of OX 40.

The engineered cells are infected with LSZR lacZ virus pseudotyped with scFv specific for OX 40 expressing cells. Infection of such cells is compared to Hela cells and Namalwa B cells not expressing OX 40. Similarly, a retroviral vector is created using the murine OX 40 cell surface marker to express murine OX 40 in the 70Z/3 pre B line. Engineered 70Z/3 cells are infected with LSZR lacZ virus pseudotyped with scFv and OX 40 ligand fusion vectors specific for cells expressing murine OX 40.

The previously described T cell clones are transduced with retroviruses containing the OX 40 ligand and with retrovirus envelope expressing an irrelevant protein region (from lacZ). Efficiency of transduction is assessed by PCR, as well as biologic readout of the appropriate cytokines.

A retroviral envelope is constructed capable of specifically targeting retroviruses to OX-40 expressing cells. The murine and human OX40 ligand determinant regions are isolated by PCR using an appropriate primer combination from the 5'-CAGGTATCACATCGGTAT until GGTGAATTCTGTGTCCTT-3' (a 398 nucleotide region from human OX-40 ligand and 5'-CAACTCTCTTCCTCTCC until AAC CAA GTA CCA CTG-3' (a 450 base pair region from murine OX-40 ligand). These regions are incorporated into xenotropic and ecotropic envelope acceptor vectors, respectively, to provide in frame fusion for expression of fusion protein. The resulting vector clones are sequenced using oligonucleotides across junction areas at the 5' and 3' junctions, respectively.

The constructs are transiently expressed in 293 cells to allow detection of envelope determinants by FACS. The soluble OX-40 recombinant fusion protein (106Ag fused

to the constant region of human IgG1 (106-Ig)) is used to stain cells as primary anti-ligand determinant. Secondary antibody is a goat anti-human IgG1 PE-conjugate. Transfected cells are also stained with 83A25 primary anti-retroviral envelope and a secondary fluorescein conjugated goat anti-rat IgG. 83A25 is a rat IgG2b that detects murine ecotropic and amphotropic envelope protein expression on cells. The percentage of cells positive for the OX-40 ligand determinant and ecotropic envelope determinant are determined. Cells are also co-stained to check for correlated expression of the ligand and envelope determinants.

The fusion construct expressing retroviral envelope protein fused to the OX 40 ligand is called pEnv:OX 40L. Using these constructs designed to direct retroviral infection to OX 40 cells, their genetic targeting capacity is tested with artificially-created cell lines. LacZ and GFP reporter genes are established as episomes (LZRS retroviral vectors) in the BOSC23 cell line, thus creating BOSC23:LZRS- lacZ and BOSC23:LZRS-GFP. To test the pEnv:OX-40L targeting construct, pEnv:OX-40L is transiently transfected into the BOSC23:LZRS- lacZ and BOSC23:LZRS-GFP cell lines. 48 hours later supernatant from these cells is used to infect human lines (HeLa and Namalwa) engineered to express OX-40 receptor. Infection is done in comparison to Hela cells and Namalwa B cells not expressing OX-40. Titres are established as compared to infection of NIH 3T3 cells as baseline.

A stable cell line constitutively expressing the targeting fusion polypeptide pEnv:OX 40L is established. The pEnv:OX 40L construct is linearized with an appropriate restriction enzyme and co-electroporated with a PGK-blasticidin construct (Funakoshi Co. LTD, Tokyo, Japan) into BOSC23 cells. Two days after electroporation, the cells are cultured in the presence of 5 μg/ml blasticidin,

to kill those BOSC23 cells that have not stably integrated the blastocidin construct PGK-blasto.

Using the soluble OX-40 recombinant fusion protein (106Ag fused to the constant region of human IgG1 (106-Ig)) cells are stained for the ligand determinant expressed as the env-OX 40 fusion. Secondary antibody is a goat anti-human IgG1 PE-conjugate. FACS is used to clone those cells stably expressing pEnv:OX-40L. Clones are grown in 60 mm dishes and restained for the ligand determinant. A clone is selected that stably expresses pEnv:OX 40L in a uniform distribution peak as determined by FACS. The line is called BOSC-Env:OX 40L. BOSC-Env:OX 40L is transfected with LZRS-GFP or LZRS-LacZ and stable episomes are established by puromycin selection. Similarly, LZRS-cytokine vectors are established in BOSC-Env:OX 40L lines.

Retroviral supernatant from these lines is used to infect activated cells. Simple kinetics to document the upregulation of OX 40 following antigen activation and to assess the level and time of expression of OX 40 following antigen stimulation are done using FACS analysis of the cell clones and the monoclonal antibodies reactive to OX 40 at various time periods following antigen-induced activation.

The cell clones are transduced with retroviruses containing the OX 40 ligand and with retrovirus envelope expressing an irrelevant protein region (from lacZ). Efficiency of transduction is assessed by PCR, as well as biologic readout, of the appropriate cytokines. This is assayed using lacZ reporter genes delivered to cell clones, and using viruses expressing dominant negative polypeptides or anti-TNF. Using the same approach, a xenotropic envelope packaging line expressing AKR xenotropic envelope and MMULV gag-pol is constructed. This line is used for targeting murine cells. The murine OX-40 ligand is cloned

into a xenotropic envelope construct and treated as for the human, except for cloning into a xenotropic packaging cell line.

VI. Targeted gene delivery in NOD mice *in vivo*.

The OX 40 retroviral gene transduction system is used to deliver genes to $CD4^+$ $OX\ 40^+$ T cells in insulitis lesions of NOD mice *in vivo*. Retroviruses of the murine Type C MMuLV and related classes employ an unusual envelope polypeptide structure. Although encoded by a single polypeptide chain, the envelope protein is cleaved during virion formation in an extracellular region to reveal a fusogenic motif attached to the virion lipid bilayer through a transmembrane domain. The cleaved N terminal region remains associated with the fusogenic region by electrostatic and disulphide bonds. Commercial equipment is available to concentrate virus via dialysis. The episomal production technique rapidly produces very large volumes of high titre retrovirus (10^6-10^7 per ml).

The volume and titre of retrovirus required to infect a mouse by direct injection is as follows. The volume of blood in a mouse (the volume into which the virus must disperse after injection) is approximately 2 ml at the upper limit (based on a fluid volume estimate of 8.8% for an average mouse with a weight of 45 grams and blood volume estimate to be 50% of the fluid volume). To achieve high efficiency infection of T cells requires a titer of at least 10^7 retrovirus per ml of blood. No more than about 0.1 ml of fluid can be injected into a mouse every 6 hours. Therefore, virus titers of at least about 5×10^8 per ml are used. Dialysis or chromatography on a nickel column is used for concentration.

Retrovirus are produced in one liter quantities by the method of episomal stabilization of the producer construct. As described above, retroviral backbones

expressing genes of interest are inserted into the EBV/EBNA vector. Constructs are transfected into BOSC23, Bing or xenotropic retargeting lines as prepared above. Selection for puromycin is applied to establish the vector in the producer cell and cells are split continuously at two to three day intervals until ten 150 mm plates are at confluency. Each plate carries 50 ml media (at the virus collection point it is changed to be puromycin free), and virus excretion is allowed to proceed for 48 hours. Virus supernatant is collected, filtered and concentrated in dialysis bags placed on dry sephadex beads. Up to 50 fold or higher concentration can be achieved in this manner.

Virions are enriched from the growth media by calcium mediated precipitation, followed by sequential washing steps with a buffer containing EDTA (as described in Morling and Russell, *supra*.)

Using the OX 40 ligand retroviral constructs, concentrated virus preparations are made for transduction of NOD mice *in situ*. Concentrated virus to be injected includes 1) OX 40 retargeted xenotropic envelope, 2) anti OX 40 scFv retargeted xenotropic envelope, 3) xenotropic envelope control. Retrovirus in the first tests express lacZ or GFP (green fluorescent protein). To provide an optimal target population for tests of *in vivo* infection, 1×10^6 IDDM inducing T cells from the CD4' disease inducing T cell clones previously described are stimulated with antigen and APC, and injected into mice 24 hours after stimulation. 24 hours later, concentrated virus is injected, 100 μl of virus concentrated to 5×10^8 per ml is injected into the tail vein of a recipient mouse. Three hours later the procedure is repeated. Mice are tested by histopathology of islets for lacZ expression. Total splenic cells are tested for lacZ expression by FACS and by histopathology to determine specificity of infection. Some of the mice are sacrificed three weeks after infection, and

T cells from the islet region, from spleen, and from control regions (liver, thymus) are isolated and cultured with APC and IDDM specific antigen. T cells cultured from these sources are assayed for retroviral expression of lacZ or GFP by FACS and costaining for CD4.

"Naive" NOD mice at 120 days are injected with concentrated lacZ or GFP expressing virus pseudotyped with either 1) OX 40 retargeted xenotropic envelope, 2) anti OX 40 scFv retargeted xenotropic envelope, 3) xenotropic envelope control again. 100 μl of virus concentrated to 5 x 10^8 per ml is injected into the tail vein of a recipient mouse. Three hours later the procedure is repeated. After three weeks, mice are sacrificed and analyzed for retroviral gene transfer by culturing specific native T cells, histopathologic analysis for lacZ or GFP expression, and FACS analysis of lacZ or GFP in costaining for CD4. Control virus used is standard xenotropic virus with noretargeting envelope.

Mice are injected with concentrated virus stocks expressing down-regulatory cytokines under constitutive control or inducible control. Mice injected with cytokine expressing viruses are followed by histopathology and blood sugar analysis for prevention of IDDM. Islet localized OX 40 positive T cells are tested for cytokine expression by PCR and ELISA.

Dominant negative mutants of NF-kB or IkB are introduced via retroviral-mediated gene transfer into diabetogenic Th-1 CD4+ T cells. Mice are injected with concentrated retroviral stocks expressing the dominant negative polypeptides and anti-TNF antibody. Mice injected with these concentrated viruses are followed by histopathology and blood sugar analysis for prevention of IDDM. Similarly, islet-localized OX 40 positive cells are tested for dominant negative expression by direct antibody

staining of fixed cells, histopathology, and for secretion of anti-TNF.

It is evident from the above results that the subject invention provides for retroviral compositions that act to specifically change the phenotype of activated T cells to down-regulate the relase of pro-inflammatory cytokines.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Board of Trustees of the Leland Stanford Junior University

(ii) TITLE OF INVENTION: Compositions and Their Uses for Transfer of Down-Regulatory Genes into Cells Associated with Inflammatory Responses

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Fish & Richardson P.C.
(B) STREET: 2200 Sand Hill Road, Suite 100
(C) CITY: Menlo Park
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94025

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Sherwood, Pamela J.
(B) REGISTRATION NUMBER: 36,677
(C) REFERENCE/DOCKET NUMBER: 06037/046001

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415) 322-5070
(B) TELEFAX: (415) 854-0875

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 177 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGGCGCGTT CAACGCTCTC AAAACCCCTT AAAAATAAGG TTAACCCGCG AGGCCCCCTA 60

ATCCCCTTAA TTCTTCTGAT GCTCAGAGGG GTCAGTACTG CTTCGCCCGG CTCCAGTCTC 120

GAGGGATCCA TGGCACACCT GACCCCTCAT CAAGTCTATA ATATCACCTG GGAGGTA 177

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Arg Ser Thr Leu Ser Lys Pro Leu Lys Asn Lys Val Asn Pro
1 5 10 15

Arg Gly Pro Leu Ile Pro Leu Ile Leu Leu Met Leu Arg Gly Val Ser
20 25 30

Thr Ala Ser Pro Gly Ser Ser Leu Glu Gly Ser Met Ala Glu Val Thr
35 40 45

Pro His Gln Val Tyr Asn Ile Thr Trp Glu Val
50 55

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 180 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGGAAGGTC CAGCGTTCTC AAAACCCCTT AAAGATAAGA TTAACCCGTG GGGCCCCCTA 60

ATAGTTATAG GGATCTTGGT GAGGGCAGGA GCCTCGGTAC AACGTGACAG CCCTCACCAG 120

CTCGAGGGAT CCAGCGGCCG CGTCTTCAAT GTCACTTGGA GAGTTACCAA CCTAATGACA 180

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Glu Gly Pro Ala Phe Ser Lys Pro L u Lys Asp Lys Ile Asn Pro
1 5 10 15

Trp Gly Pro Leu Ile Val Ile Gly Ile Leu Val Arg Ala Gly Ala Ser
20 25 30

Val Gln Arg Asp Ser Pro His Gln Leu Glu Gly Ser Ser Gly Arg Val
35 40 45

Phe Asn Val Thr Trp Arg Val Thr Asn Leu Met Thr
50 55 60

WHAT IS CLAIMED IS:

1. A retrovirus comprising:

an inflammatory down regulatory sequence encoding a protein capable of decreasing local inflammatory responses in a mammalian host; and

a promoter sequence upstream of said down regulatory sequence;

wherein upon introduction of said retrovirus into a host cell, said down-regulatory sequence is expressed.

2. A retrovirus according to Claim 1 wherein said down-regulatory DNA sequence encodes a down-regulatory cytokine.

3. A retrovirus according to Claim 1 wherein said down-regulatory DNA sequence encodes a blocking protein of a pro-inflammatory cytokine.

4. A retrovirus according to Claim 3, wherein said blocking protein is an antibody.

5. A retrovirus according to Claim 1 wherein said down-regulatory DNA sequence encodes a dominant negative signaling molecule.

6. A retrovirus according to Claim 1, comprising chimeric retargeting envelope protein (CREnv) specific for an inflammation associated cell.

7. A retrovirus according to claim 1, wherein said retrovirus is self-inactivating, and said promoter is an exogenous promoter induced in activated T cells.

8. A retrovirus according to claim 1, wherein said retrovirus is a provirus integrated in the genome of a cell capable of localizing to a site of inflammation.

9. A method of decreasing the disease associated inflammation in a mammalian host, the method comprising:

administering a retrovirus according to Claim 1 to said patient, in an amount sufficient to decrease said inflammation.

10. A method according to Claim 9, wherein said disease is an autoimmune disease.

11. A method according to Claim 9, wherein said disease is caused by infection.

Figure 1 A

Figure 1 B

Figure 1 C

Figure 1 D

Figure 2

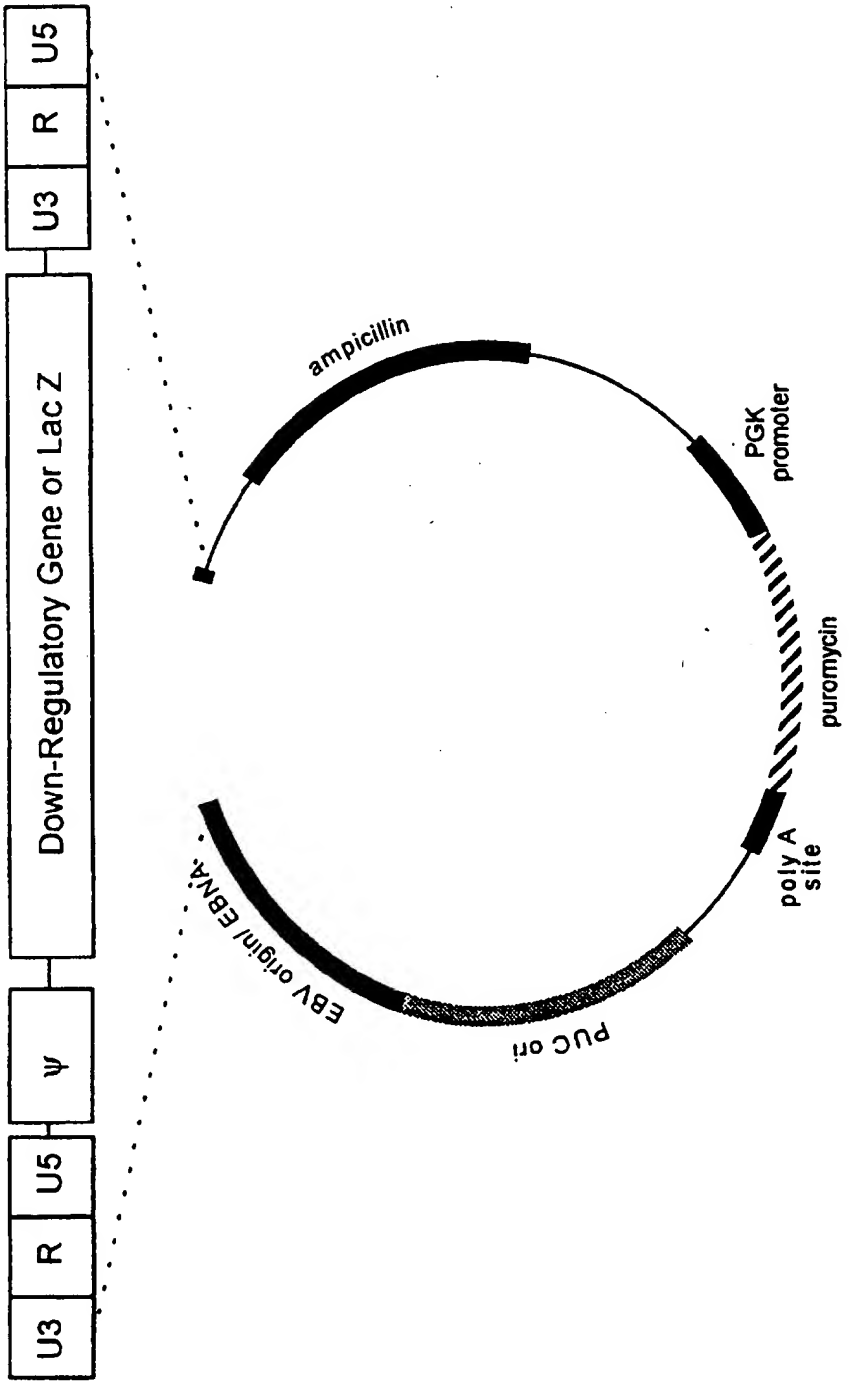

U3
R
U5
ψ
U5
R
U3
Down-Regulatory Gene or Lac Z
ampicillin
PGK promoter
puromycin
poly A site
EBV origin/ EBNA
PUC ori

Figure 3 A

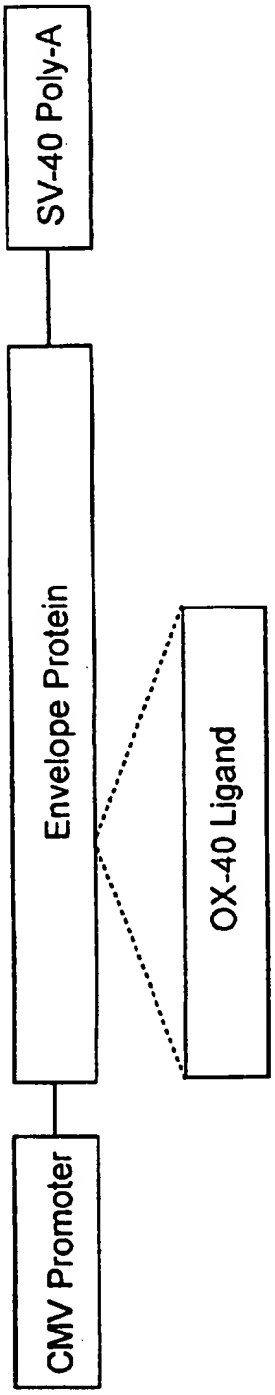


Figure 3 B

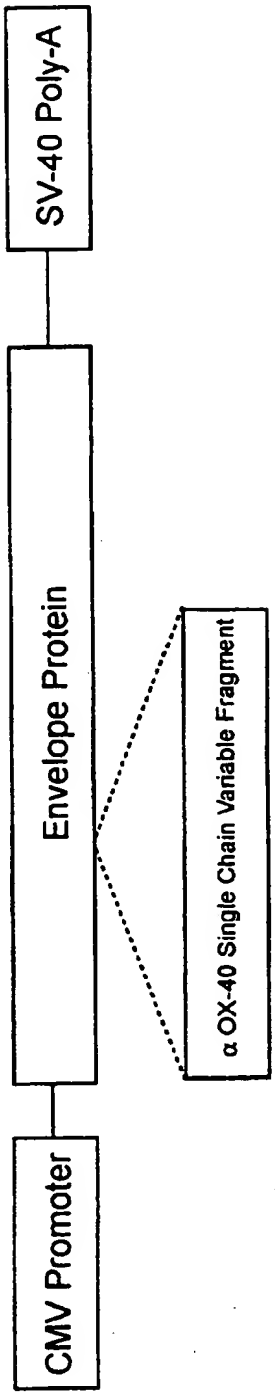


Figure 3 C

Leader peptide
atg gcg cgt tca acg ctc tca aaa ccc ctt aaa aat aag gtt aac ccg cga ggc ccc cta
Met ala arg ser thr leu ser lys pro leu lys asn lys val asn pro arg gly pro leu

Leader peptide ←|→ Mature Envelope

atc ccc tta att ctt ctg atg ctc aga ggg gtc agt act gct tcg ccc ggc tcc agt
ile pro leu ile leu leu met leu arg gly val ser thr ala ser pro gly ser ser

Xho I Bst EII
CTC GAG GGA TCC ATG GCA GAG GTC ACC cct cat caa gtc tat aat atc acc tgg gag gta
LEU GLU GLY SER MET ALA GLU VAL THR pro his gln val tyr asn ile thr trp glu val

Replacement Region for Fusion

Figure 3D

Leader Peptide
atg gaa ggt cca gcg ttc tca aaa ccc ctt aaa gat aag att aac ccg tgg ggc ccc cta
Met glu gly pro ala phe ser lys pro leu lys asp lys ile asn pro trp gly pro leu

Leader peptide ←|→ Mature Envelope

ata gtt ata ggg atc ttg gtg agg gca gga gcc tcg gta caa cgt gac agc cct cac cag
ile val ile gly ile leu val arg ala gly ala ser val gln arg asp ser pro his gln

CTC GAG GGA TCC AGC GGC CGC gtc ttc aat gtc act tgg aga gtt acc aac cta atg aca
LEU GLU GLY SER SER GLY ARG val phe asn val thr trp arg val thr asn leu met thr

Replacement Region for Fusion

Figure 4A

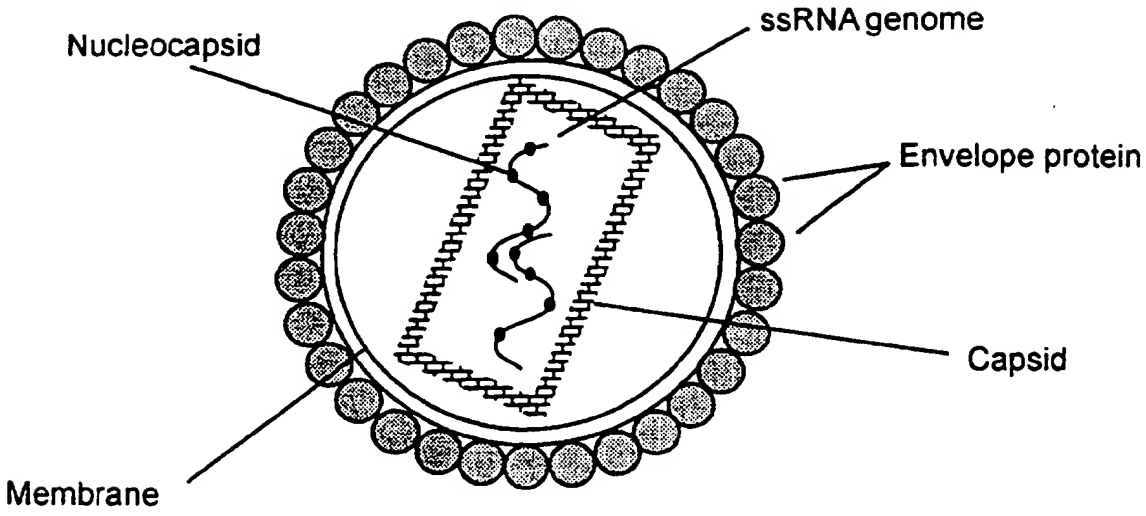


Figure 4B

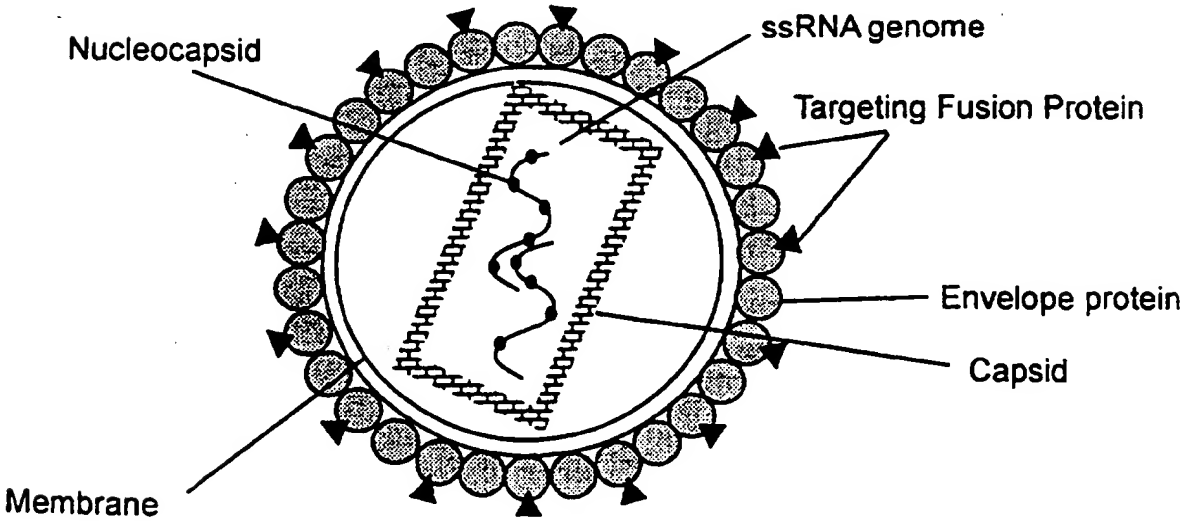


FIGURE 5

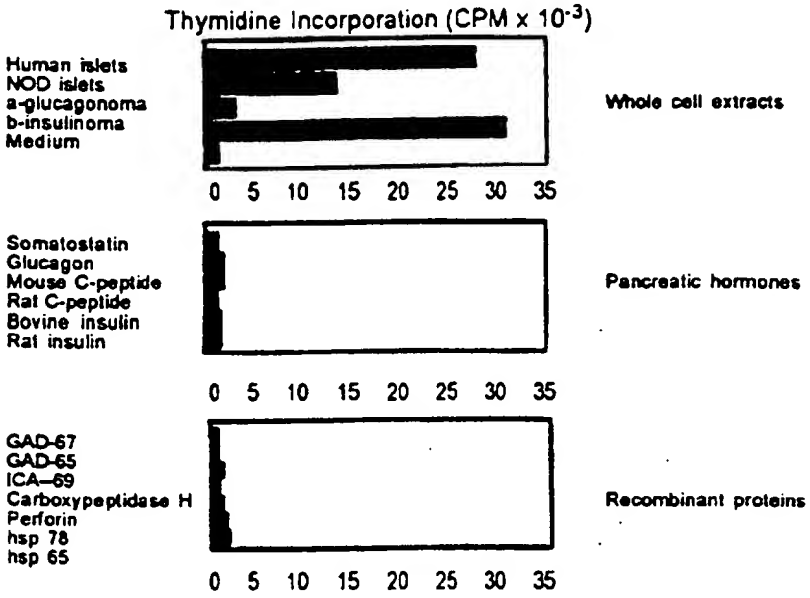
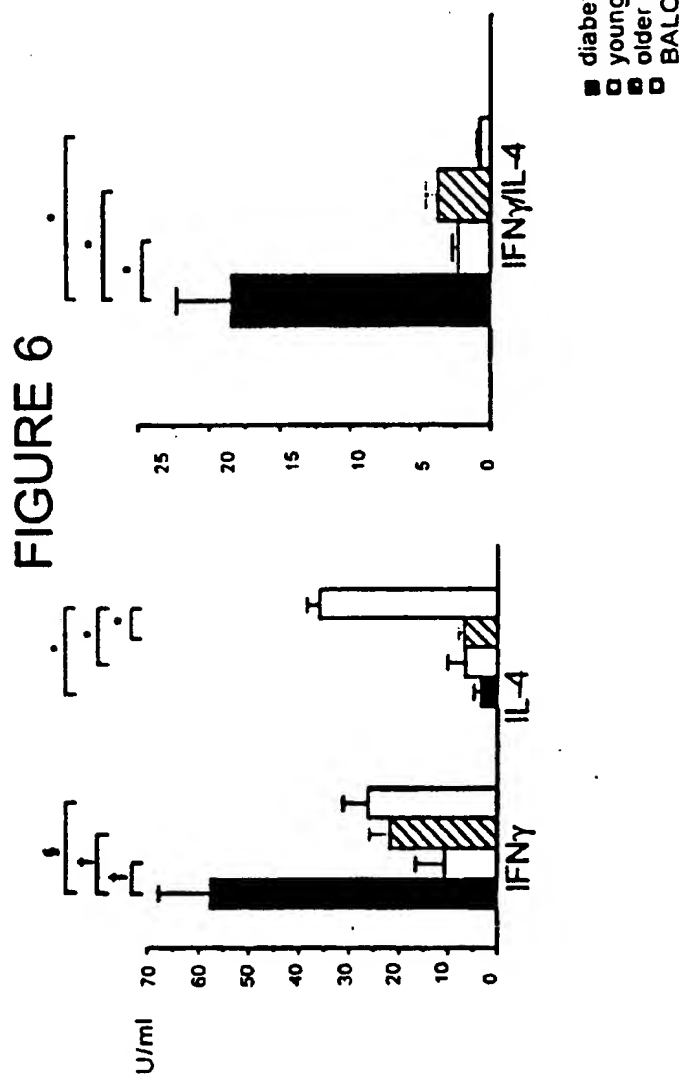

Thymidine Incorporation (CPM x 10-3)
Human islets
NOD islets
a-glucagonoma
b-insulinoma
Medium
0 5 10 15 20 25 30 35
Whole cell extracts
Somatostatin
Glucagon
Mouse C-peptide
Rat C-peptide
Bovine insulin
Rat insulin
0 5 10 15 20 25 30 35
Pancreatic hormones
GAD-67
GAD-65
ICA-69
Carboxypeptidase H
Perforin
hsp 78
hsp 65
0 5 10 15 20 25 30 35
Recombinant proteins

FIGURE 6



* p<0.001, † p<0.01, § p<0.05

FIGURE 7

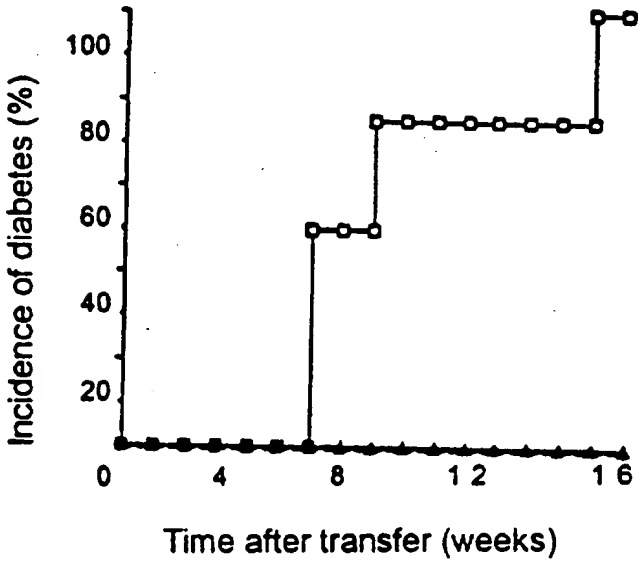

Incidence of diabetes (%)
100
80
60
40
20
0
4
8
12
16
Time after transfer (weeks)

Figure 8A

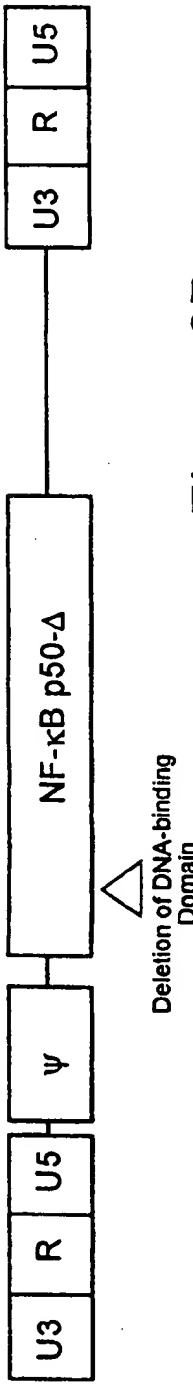


Figure 8B

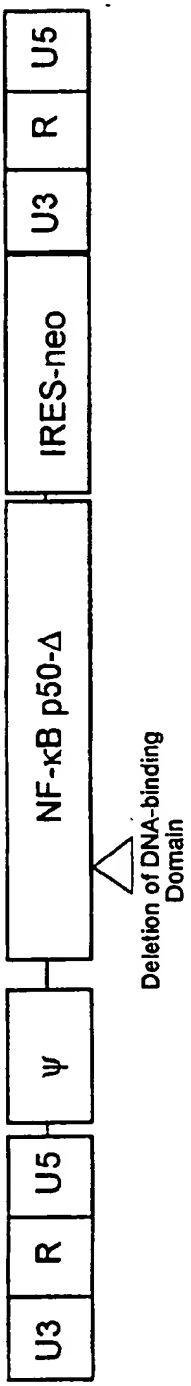


Figure 8C

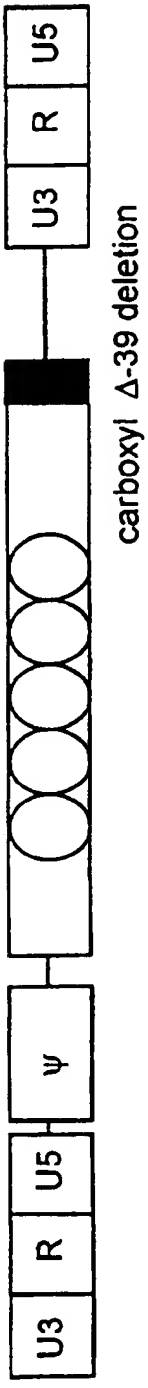


Figure 8C

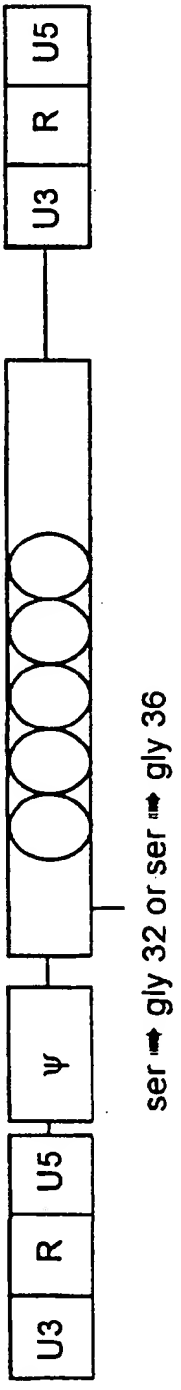
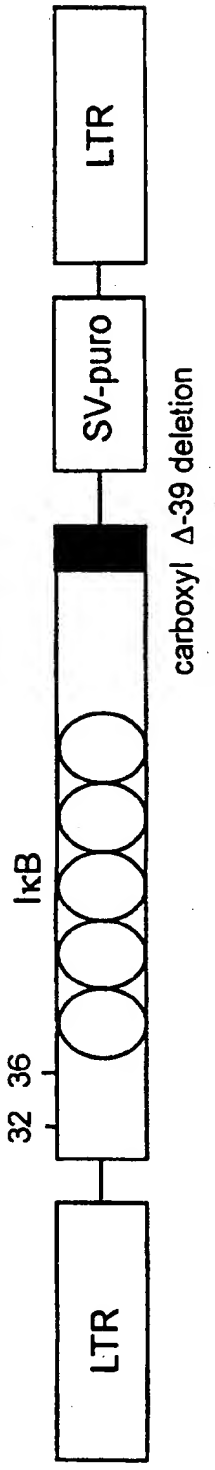


Figure 9



Western Blot Analysis of Mutant IκB-α Expression

Figure 10A

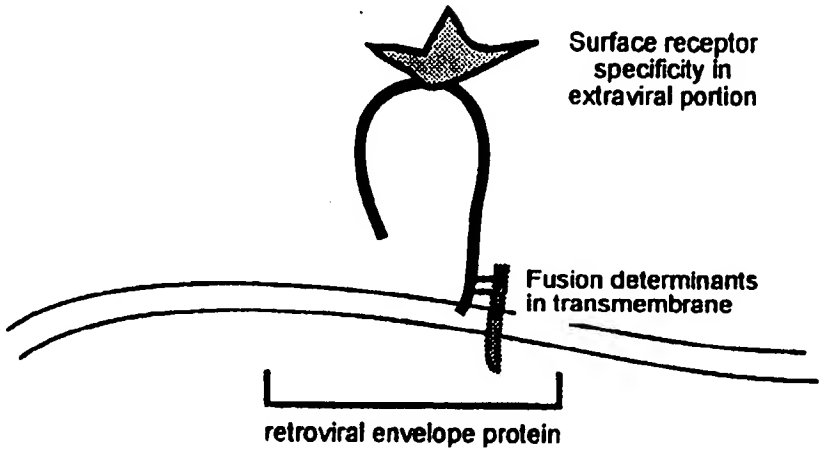


Figure 10B

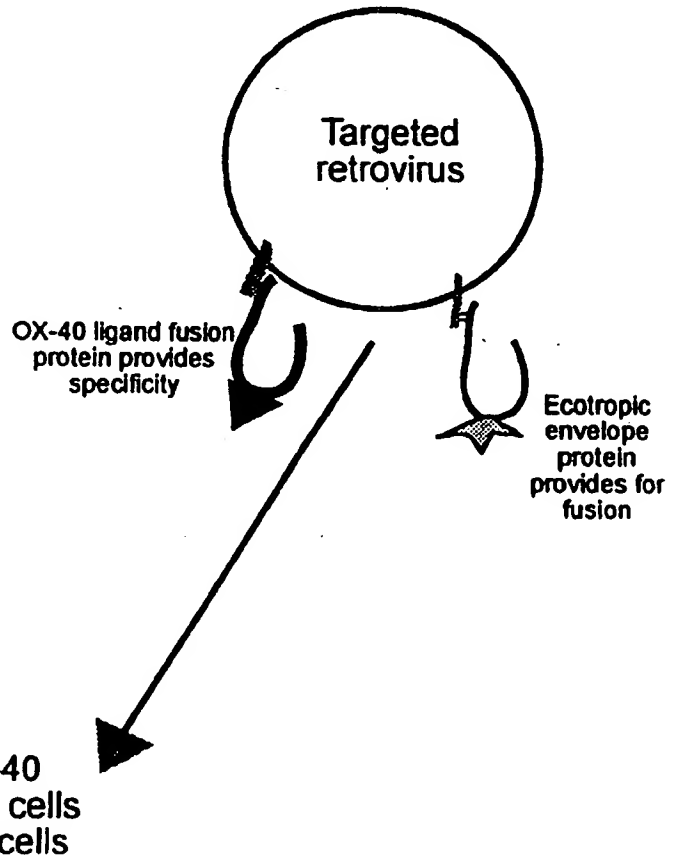
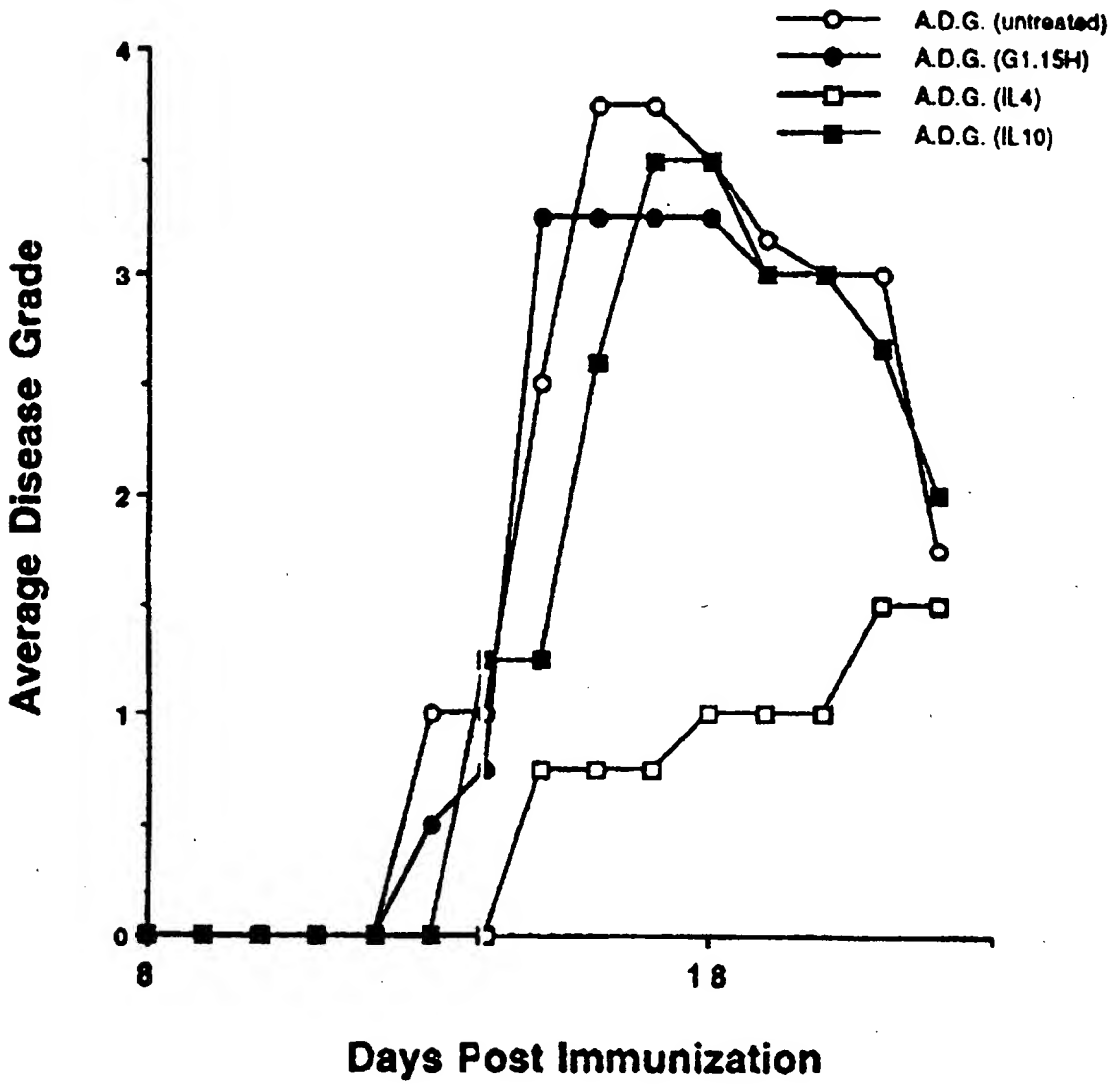


FIGURE 11


A.D.G. (untreated)
A.D.G. (G1.15H)
A.D.G. (IL4)
A.D.G. (IL10)
Average Disease Grade
0
1
2
3
4
8
18
Days Post Immunization

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01178

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :435/320.1, 6, 69.1, 172.3; 514/44; 935/62, 57, 71, 34; 424/93.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 6, 69.1, 172.3; 514/44; 935/62, 57, 71, 34; 424/93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, APS, STN, CAPLUS, EMASE
search terms: down regulatory, t-cell, vector, dominant negative, self-inactivating

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BROWN et al. 'Retroviral Vectors.' In: DNA Cloning. Edited by D.M. Glover et al. Oxford, UK: 1995, Vol. 4, pages 113-142.	1-11
Y	JULIAS et al. E- Vectors: Development of Novel Self-Inactivating and Self-Activating Retroviral Vectors for Safer Gene Therapy. J. of Virology. November 1995, Vol. 69, No. 11, pages 6839-6846.	1-11
Y, P	SAWCHUK et al. Anti-T Cell Receptor Monoclonal Antibody Prolongs Transgene Expression Following Adenovirus-Mediated In Vivo Gene Transfer to Mouse Synovium. Human Gene Thearpy. 01 March 1996, Vol. 7, pages 499-506.	1-11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
06 MAY 1997

Date of mailing of the international search report
03 JUN 1997

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01178

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	OLSON et al. Improved Self-Inactivating Retroviral Vectors Derived from Spleen Necrosis Virus. J. of Virology. November 1994, Vol. 68, No. 11, pages 7060-7066.	1-11
Y, P	GILLIO et al. Retroviral Vector-Mediated Transfer of the Tumor Necrosis Factor alpha Gene into Human Cancer Cells Restores an Apoptotic Cell Death Program and Induces a Bystander-Killing Effect. Blood. 15 March 1996, Vol. 87, No. 6, pages 2486-2495.	1-11
Y	LACOSTE et al. Interactions Between HTLV-I Tax and NF-kappa B/Rel Proteins in T-Cells. Leukemia. April 1994, Vol. 8, Supl. 1, pages S71-S76.	1-11
Y	IVANOV et al. Pleiotropic effects of Bcl-2 on transcription factors in T cells: Potential Role of NF-kappa B p50-p50 for anti-apoptotic function of Bcl-2. International Immunology. November 1995, Vol. 7, No. 11, pages 1709-1720.	1-11
Y	NABEL et al. Immunotherapy for Cancer by Direct Gene Transfer into Tumors. Human Gene Therapy. 1994, Vol. 5, pages 57-77.	1-11
Y	US 5,399,346 (ANDERSON et al.) 21 March 1995 (21.03.95), entire patent.	1-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01178

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 5/00, 15/00, 15/63, 15/79, 15/24, 15/09; A61K 48/00; C07H 21/00